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VITAMINOLOGY

The Chemistry and Function
of the Vitamins

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FOREWORD

I first met Dr. Eddy in 1916. At that time I had a laboratory at the old Loomis Building, then a part of Cornell Medical School. He walked in one day and as I explained to him some nutritional data in front of the animal cages, he informed me that he intended to quit his high school teaching career and go into vitamin research. I judge he was about 35 then and for that reason I did not take his decision too seriously. But shortly afterward, he obtained the very enviable position as professor of biochemistry at Teachers College, Columbia University and by that time we all knew that this had become his life work.

Indeed, as a teacher, leader in research, and popularizer of the science of nutrition he became one of the pioneers in the field. To exemplify the importance he attained, it suffices to mention that the important work of R. R. Williams on Vitamin B_1 was accomplished in his department with the aid of such men as Waterman, Keresztesy and Gurin as well as his own highly valuable scientific publications with his collaborators.

Since the time I first met Dr. Eddy we have remained loyal friends through all these years and when he and the publishing house of Williams and Wilkins asked me to write a foreword to the present publication, I considered it a pleasure and my duty to comply.

I have now before me a copy of the first edition of Dr. Eddy's Vitamine Manual of the year 1921 and a page proof of his Vitaminology of the year 1949. One is amazed at the wealth of information accumulated during this lapse of time. The methods of scientific investigation have changed radically and the number of highly qualified workers increased to such an extent, that a problem that took 25 years to bring to a conclusion is liquidated in a year or two, the product being placed at the disposal of research and made available to the public. Each such new advance opens up new lanes of research and new vistas for the future.

Dr. Eddy's book is particularly interesting in this respect. It possesses the vast subject matter organized in a manner that will satisfy a large number of inquirers. I was very much interested in his treatment of vitamins as the integral building stones of the various enzyme systems. For one I know that I learned a great deal and I trust, that will also be the case with other readers.

A few words as to future outlook of Vitaminology. I shall not talk here about the relationship of vitamins to hormones and enzymes, about the importance of trace elements, subjects which have been extensively treated before. I would like to draw attention to the fact, that the vitamins, which are present in our foods in relatively large amounts, have been mostly

accounted for and even synthesized. Our task is not finished and t
remains a large number of “gamma” vitamins to be identified. W
present in minute amounts they will prove not less important. Each a
tion of these to an artificial dietary brings new deficiencies to our attent
The time is yet remote, in spite of some optimistic findings of the last
years, that an animal will subsist, become adult, and multiply on a mixt
of fully purified ingredients.

For many years I was concerned with another problem of the future
the reading of Dr. Eddy's book has stimulated me to write a few rema
about it. In the middle of the 20's I reported isolation of a crystall
material from a then rather crude insulin. This substance injected i
rabbits caused diabetes in a week and might have been identical with an
loxan derivative. I was struck with the fact that the same organ—pancrea
should contain both the diabetogenic and anti-diabetic factors. M
recently I have isolated from natural sources crystalline as yet unidentifi
fractions; some increasing, others inhibiting the formation of new neoplas
tissue. The study of degenerative pathological changes of old age (a subj
not yet popular naturally with younger workers and for which older h
retired workers are ill equipped), may well belong to a future chapter
vitamin research. I feel that a proper balance of stimulating and inhibiti
factors in our dietary, with the possibility of correcting such ill-balance
the selection of the right foods or specified isolated principles, may elu
date quite a few pathological states, which at present baffle the investigat

Dr. Eddy's book describes methods of approach for many such problen

CASIMIR FUNK

New York, August 29th, 1949.

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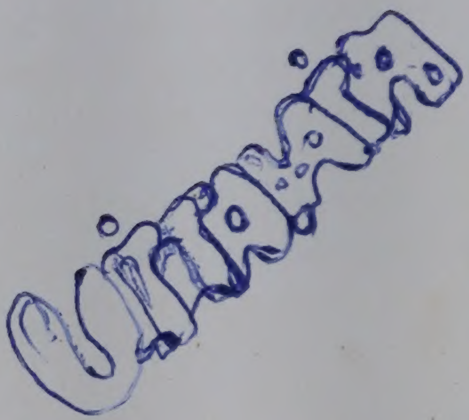

CHAPTER I. INTRODUCTION: WHAT IS A VITAMIN?

The development of the classification of certain organic substances under the name of vitamins really began as far back as 1881 when Lunin (1) reported that while mice could be satisfactorily nourished under laboratory conditions with milk they could not be so nourished on purified proteins, fats, carbohydrates and minerals and water, indicating the milk must contain substances outside that group essential to nutrition.

Other sporadic reports in the interim suggested existence of such factors, but vitamin research workers generally agree that credit is due to Gowland Hopkins (2) in 1906 for first demonstrating that normal food and normal nutrition contain essential factors not classified at the time as nutrients. Hopkins did not call these factors vitamins (that name came later), but suggested the term "accessory factors".

To Casimir Funk (3) we owe the name which in 1911 he spelled *vitamine* with a terminal *e*. This was based on his belief at the time that the crystalline product which he isolated from rice polishings and later from yeast was a chemical "amine". Since it was also a means of preserving the life of beri-beri patients, he prefixed amine with the Latin word for life (vita) and called it the life-amine or "vitamine".

To Funk also, we owe the first formulation of the so-called Vitamin Hypothesis of Deficiency Disease (4), the prediction that certain diseases such as beri-beri, scurvy, pellagra, etc., would be shown to be a result of vitamine deficiency. To him we owe the term "avitaminosis" to express in one word vitamin deficiency disease.

Sixteen organic substances or groups of substances representing products generally classified today as vitamins, and with established chemical identity and structure, have been treated in the following chapters. There is no reason to believe that this list is a complete presentation of vitamins, and in Chapter XIX are listed some other compounds that have already been postulated as candidates for vitamin inclusion—postulated but not yet chemically identified.

In checking these vitamins it will be noted that Funk's terminal *e* is now omitted, and that letter designations are used for some vitamins while others are presented under chemical names. This is evidence that vitamin nomenclature, like vitamin research, is still in process of development. The chapter on Vitamin A explains how the discovery by McCollum and Mendel of this factor, lacking of an amine group, led McCollum to reject calling it a vitamine and to suggest instead the phrase "unidentified dietary factor, fat-soluble A". Also this chapter explains how in 1920 Drummond suggested getting rid of this rather cumbersome designation by retaining the lett designation, but omitting the terminal *e* and making the term vitamin avoid implication of amine content. Thus the designations as vitamin A, C, etc. arose.

In the earlier days of vitamin research presence and action of the s called vitamins was established by physiological response; chemical iden fication of the compounds came much later. In the period before chemic identification, designation was by letter or by a descriptive term indicati relation to specific disease; "anti-neuritic vitamin" for vitamin B, "an scorbutic vitamin" for vitamin C, etc.

With chemical identification it seemed more significant to discard th letter system and to use a name that expressed the chemical nature of th vitamins. Vitamin B_1 became thiamine; B_2 became riboflavin. This tren has continued and in the case of the more recently isolated vitamins th chemical name alone has been used. However, a mixture of the system continues; vitamins A and D are still more usually so designated whi folic acids, choline, and the tocopherols are usually referred to today b that name alone.

However, regardless of the method of designating a single substanc they are grouped together as *vitamins*; just what does the term vitam imply?

Schopfer in his Plant Vitamins (5) defines a growth factor of vitam nature as:

> An organic substance, the need of which results from the loss of capacity to sy thesize it; whose action is catalytic (i.e. in small amounts), quantitative, and mar edly specific."

Schopfer's "loss of capacity to synthesize" once constituted a means distinguishing a vitamin from a hormone, but later evidence rather damage this distinction when it was found that many animals regularly synthesize their requirement of particular vitamins.

His requirement that to be a vitamin it must act in small amounts generally accepted, but just how much is a little? On that basis has aris the question as to whether inositol and choline be accepted as true vitamin

There has also grown up a tendency to admit to the vitamin classificati only substances that are essential to animal nutrition. As noted in Chapt VI, R. J. Williams has made the suggestion that this restriction to effe on animals be retained as a criterion for inclusion with vitamins but th substances that act similarly on other organisms be classed as nutrilit at least until their effect on animals is established.

The vitamin classification then, has been largely a matter of convenien rather than a sharply defined term. It is a functional term rather than chemical classification. It appears superior to Hopkins' original "accessory factor" since these substances are not only accessory to the so-called nutrients but actually *essential* to nutrition, an essentiality that the term "accessory" does not necessarily imply.

If then qualification as a vitamin is to be a functional classification, the important objective in vitamin research today is not simply chemical identification of the compounds but determination of just what functions each vitamin has. For that reason attempt has been made in the following chapters to stress what functions appear established, what have been suggested, and what remain for further investigation.

The present bibliography of vitamin research has become so extensive in each vitamin field as to make impossible complete presentation of contributions or credit to contributors. The papers selected in this text are primarily to lead the student to the problems. They make no pretense to complete coverage of any field, but it is hoped that in following up the fields suggested the student will find guidance for his own efforts in vitamin research.

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CHAPTER II. VITAMIN A

Section I. Functions of Vitamin A

Vitamin A owes its position in the alphabetical designation of vitamin to Dr. E. V. McCollum. McCollum and Davis (1) named the growth facto which they isolated from egg yolk and butter fat "unidentified dietar factor fat-soluble A". Later, when its position as a vitamin was establishe Drummond (2) suggested the retention of the letter designation prefixe with the word vitamin; vitamin spelled without a terminal *e* to avoid th implication that it was an amine.

The first property of vitamin A to be established was its ability to stimulate growth. The biological assay for vitamin A potency still employs effect on animal growth for its detection.

Subsequent study revealed other functions, namely ability to help maintain normal body coverings and linings, and normal acuity of vision.

The Council on Pharmacy and Chemistry of the American Medical Association has listed the following as allowable therapeutic claims for vitamin A:

1) Vitamin A is a specific for cure and prevention of xerophthalmia (dry eye disease), nyctalopia (night blindness), and hemeralopia (day blindness).

2) Vitamin A is essential to the normal structure and behavior of epithelial tissue, e.g. the epithelium covering the skin, and forming the lining of the nasal, sinus, and respiratory tract, mouth, pharynx, entire digestive tract and the genito-urinary tract. It prevents follicular hyperkeratosis of the skin.

3) Vitamin A may be of value in resistance to infections but only when there has been an exhaustion of body reserves or inadequacy of intake.

4) Vitamin A is a growth factor.

Other functions of vitamin A have been reported. Such claims and the nature of the vitamin's action are discussed in the following pages.

Vitamin A as a Growth Factor

The standard bioassay method for vitamin A employs the following procedure. Young rats are given a basal diet complete in all known ingredients (nutrients, minerals and vitamins) with the exception of vitamin A. On such a diet growth (expressed as weight gain) ceases after about 35 days. Growth is promptly restored at this time by feeding vitamin A and the rate of growth is proportional to the amount of vitamin A fed.

The original definition of a vitamin A unit was the amount necessary to produce a weight gain in young rats of 3 grams per week. This definition was improved by the use of a vitamin A reference standard of known potency. In the United States this was originally a cod liver oil supplied for assays by the U. S. Pharmacopoeia Committee. It was supposed to contain exactly 850 so-called U.S.P. units per gram. With the discovery of the provitamin A known as beta carotene, it became possible to use this as a reference standard and today the U.S.P. or International unit is defined as an amount of vitamin A equivalent in physiological effect to .0006 mg. of beta-carotene. Recently the U.S. Pharmacopoeia has adopted as a reference standard a stable form of vitamin A ester (vitamin A acetate).

The growth effect of vitamin A has therefore been used to not only detect the presence and amounts of vitamin A but also to provide a method of expressing potency in either units or in actual weights of vitamin. In brief, a U. S. P. or International unit of vitamin A is .0003 mg. in weight, an amount equivalent in effect to .0006 mg. of beta-carotene. (See p. 20.)

Growth or weight maintenance is not, however, the only basis for estimating vitamin A adequacy in diet. Dietary deficiency can and does produce visible symptoms other than growth failure or weight loss.

Xerophthalmia

As early as 1913 Osborne and Mendel (4) reported that: "A type of nutrition deficiency exemplified in a form of infectious disease, prevalent in animals inappropriately fed, is speedily alleviated by the introduction of butter fat into the experimental rations." They further stated that they had: "uniform success by substituting cod liver oil for a portion of the lard in our standard diet . . . . not only was growth resumed, but all evidence of malnutrition, *especially of the eyes* promptly disappeared."

The affection of the eyes reported by these investigators has come to be called xerophthalmia or "dry-eye disease".

It usually began with a swelling of the lids of one or both eyes followed by an inflamed and catarrhal condition of the lid linings (conjunctivae) together with a bloody or purulent discharge. The lids became scabby and sticky and often the eye became completely closed. In severe cases the inflammation extended to the cornea with perforation of the eye ball and complete blindness. The most interesting thing about it was that while it gave the appearance of an infection, it could be prevented and, if not too severe, completely and quickly cured by ingestion of vitamin A or carotene-rich foods. The name xerophthalmia came with the discovery that in acute vitamin A deficiency the flow of tears from the tear glands was blocked, permitting drying of the conjunctival and corneal surfaces.

The affection has been called opthalmia, xerophthalmia, kerato-malacia, and kerato-conjunctivitis. The latter two names owe their origin to the discovery that in this disease there is not only inflammation of the conjunctivae but also formation of keratin (horny) patches on the epitheli membranes lining the conjunctivae.

The affection was first observed in laboratory test animals but in 190 Mori (5) reported its occurrence in Japanese children, and observed tha the affection could be cured by feeding chicken livers. In 1917 Bloch (reported in Danish children a wide spread occurrence of the disease, trace to inadequate intake of butter and whole milk and curable by ingestion vitamin A rich foods.

Students of xerophthalmia cases in both laboratory animals and childre were struck by the high incidence of respiratory diseases and localized infections that frequently accompanied the eye affection.

Metaplasia

In 1925 Wolbach and Howe (7) showed that vitamin A deficiency produces histological changes in the epithelial tissues. Wolbach describes thes changes as follows:

> In the early stages of Vitamin A deficiency, areas of darkly stained epithelial cell are seen to undergo rapid growth. As they grow the underlying epithelium degenerate and is sloughed off. Islands of stratified, squamous, and keratinized patches form When this condition is treated with adequate vitamin A the process is reversed.
>
> First there is a separation of the keratinized layer and vacuoling of the cells the intermediate layer. The upper zone deteriorates. The keratinized cells are the pushed off and their places taken by the deep zone cells which are normal and no keratinized.

Sherman (8) has explained how vitamin A deficiency-induced metaplasi could lower resistance to infection in these words:

> The higher incidence of infection observed among individuals on diets low i vitamin A may be largely due to the fact that the displacement of norma by squamous epithelium implies not only a loss of local secretion, but also a loss o cilia which normally act with the secretion in cleansing the surfaces of the respirator tract by expulsion of bacteria. Plainly the normal surface thus tends to protect th respiratory system both from bacteria and from miscellaneous particles which migh cause mechanical injury and so increase the danger of the establishment of infectio by such bacteria as may not be expelled. It has also been suggested that the loca roughness and stickiness which accompanies the breaking up of normal tissue (whe it is first being replaced by squamous) may also increase the chances of lodgement o bacteria which normally would have been expelled.

Because of the infections that result from vitamin A deficiency Mellanb (9) suggested calling vitamin A the "anti-infective" vitamin. This suggestion was rejected since, unlike an antiseptic or bactericide, the vitamin doe not directly attack an infective organism. When vitamin A therapy result in a cure of an infection or its prevention it does so by maintaining or restoring the protective epithelium to normal resistance to bacterial invasion

Its value in "cold" then is not to combat the cold virus but to maintain the epithelium of the respiratory tract in a resistant condition.

Nyctalopia and Hemeralopia

Both of these terms describe a vitamin A deficiency characterized by inability to see clearly in dim light. Nyctalopia literally means "night blindness"; hemeralopia, "day blindness". The commonly used term is "night blindness" or loss of visual acuity. As early as 1921 McCollum, Simmonds and Parsons (10) attributed night blindness to persistence in diets low in vitamin A content. Explanation of how vitamin A deficiency produces this result came much later.

It is well known that the human eye operates like a camera. The eye lens focuses images on a layer of cells called the retina, which corresponds to the plate or film of the camera. But the retina differs from the photosensitive film of the camera in the ability to transmit a continuous stream of light impulses to the brain via the optic nerve. The retinal structures that accomplish this are the so-called "rods and cones" and the photosensitive pigments in the rod and cone layers. The cone layers are used in color vision; the rod layers function, as light becomes dim, to maintain clarity of vision (visual acuity). Light impulses transmitted via the optic nerve are the result of chemical changes induced by light rays in the rod and cone pigments. The rod pigment involved in visual acuity is a carotenoid pigment called rhodopsin or visual purple.

As early as 1925 Fridericia and Holm (11) produced evidence not only that ability to see in dim light depended upon the amount of rhodopsin in the rod layers but also that the suggested effect of light was to bleach this pigment. It also appeared that, before a second light impact was producible, the bleached pigment had to be restored to its original purple. For this the retina required a continuous supply of vitamin A or carotene.

It was Wald (12) however, who in the years 1935–37 worked out the details of this operation. It was shown that rhodopsin was a rose colored or purplish carotenoid pigment (maximal absorption band 500 $\mu\mu$). Impact of light bleached this pigment to a yellow or orange color, a modified pigment called retinene (maximum absorption band at 327 $\mu\mu$ in chloroform solution). In dim light part of the retinene is reversibly changed back to rhodopsin; the rest is excreted. Vitamin A from the blood stream combines with a protein to supply the depleted rhodopsin. The operation is shown graphically in figure 1. Hecht and coworkers (13) have reported that vitamin A may also be essential to cone pigments as well as to the rod pigment.

In nyctalopia, then, vitamin A deficiency deprives the retina of an essential pigment forming material. The result is loss of clarity of vision and power to adapt the eye to transition from light to dimness, loss of what is called "dark adaptation."

There are instruments for measuring the degree of dark adaptation, an such measurements have been used to determine the amount of vitamin we need to keep dark adaptation normal.

In the following section it is explained that there are several forms Vitamin A. The type found in the liver oils of marine fishes is called vitami A_1. In certain fresh water fish and some other vertebrate animals there

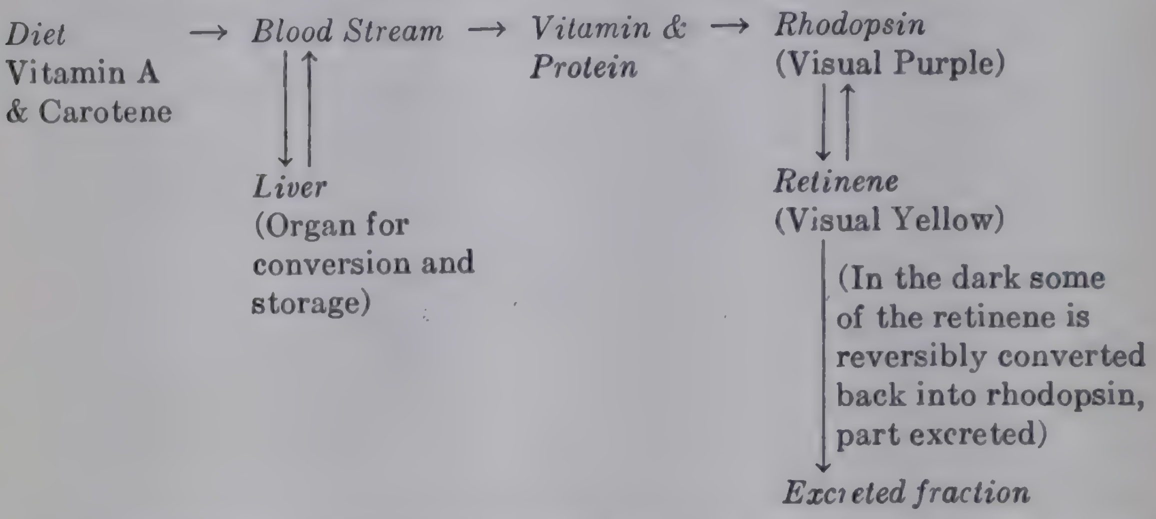
Diet
Vitamin A
& Carotene
Blood Stream
Liver
(Organ for conversion and storage)
Vitamin &
Protein
Rhodopsin
(Visual Purple)
Retinene
(Visual Yellow)
(In the dark some of the retinene is reversibly converted back into rhodopsin, part excreted)
Excreted fraction

FIG. 1. SCHEMATIC REPRESENTATION OF VITAMIN A AND THE VISUAL CYCLE

TABLE 1

Relation of retinal rod pigments to forms of vitamins A_1 and A_2

Species of animals	Name of pigment	Form of vitamin A
Marine fishes and land vertebrates..	Rhodopsin	A_1
Amphibia........................	Rhodopsin more than porphyropsin	$A_1 > A_2$
Catadromous fishes...............	Rhodopsin more than porphyropsin	$A_1 > A_2$
Ana-dromous fishes...............	Porphyropsin more than rhodopsin	$A_2 > A_1$
Fresh water fishes................	Porphyropsin	A_2
Arthropods and mollusks.........	Rhodopsin	A_1

a form called vitamin A_2. In the matter of forming rod pigments they func tion similarly but the pigments are slightly different in composition. Th rod pigment formed from vitamin A_2 and protein is called porphyropsir Substitute porphyropsin for rhodopsin and vitamin A_1 for A_2 in figure and the reactions are the same. In Table 1 is shown the distribution vitamin A_1 and A_2 in different vertebrates.

Effect of Vitamin A on Nerve Function

In 1924 Mellanby presented evidence of a nerve degeneration followin dietary deficiency of vitamin A. Wolbach and Bessey (14), however, coul find no satisfactory evidence that the degeneration of the myelin nerve sheaths was a specific consequence of lack of adequate intake of vitamin A.

It was noted, however, that during a period of vitamin A deficiency spinal and some other skeletal bones ceased to grow normally. The nerves continued to grow. As a result the central nervous system outgrew its bony vault with consequent pressure on the nerves. The effect on the nerves resulted not from their failure to get vitamin A but from pressure as a result of lack of vitamin A on bone growth.

In young dogs it was shown that when their diet was deficient in vitamin A there was overgrowth of skull bones, causing compression, twisting, and lengthening of certain cranial nerves. The resulting degenerative changes were mainly in the sensory, not the motor cranial nerves.

Therefore, it is generally conceded today that the principal effects of vitamin A deficiency are reduced growth stimulation, metaplasia of epithelial tissue, and inadequate regeneration of retinal pigments. Metaplasia effects differ in appearance with the locus of the metaplasia.

Forms of Metaplasia due to dietary vitamin A deficiency

Skin Metaplasia: Skin lesions due to vitamin A deficiency were first reported by Nichols (15) in India and Frazier and Hu (16) in China. The skin lesions took the form of a hyperkeratosis (hornifying or keratinizing of the epithelial linings of the hair follicles). This keratinization blocked the lubrication of the skin by the oil secretions, thus producing a dry skin often followed by infection and papule formation. The keratinized lining actually plugged the openings and cut off the flow of oil from the oil glands. In acute cases these plugs actually protruded, giving the skin a "toad skin" appearance (phrynoderma).

Tooth Enamel Metaplasia: It will be noted by reference to table 6 that pregnancy increases the daily requirement of vitamin A. One reason for this is that during pregnancy the teeth of the infant are in process of formation and the supply of vitamin A controls the method of enamel formation. When the supply of vitamin A from the mother's blood is inadequate, the enameloblasts are replaced by stratified keratinized epithelium. The enamel formed is thin and chalky patches appear, due to exposure of the underlying dentin layer. Such teeth in later years are more susceptible to decay. According to Wolbach and Howe (7) the odontoblasts that form the dentin may atrophy and tooth growth entirely cease in acute vitamin A deficiency of the mother's diet.

Metaplasia in the Mouth and Ears: Metaplasia of the lining of the salivary gland ducts and pus formation is one of the earliest manifestations of vitamin A deficiency in test animals such as the rat. Donnell (17) has reported an infection of the middle ear (otitis media) which he attributes to vitamin

A deficiency. He found the disease to be arrested by local use of wic carrying cod or halibut liver oil.

Metaplasia of the lining of the Gastro-intestinal tract: Bessey and Wolba (18) state that metaplasias of the gastro-intestinal lining are of rare occu rence in man as a consequence of vitamin A deficiency. Such changes hav however, been reported. Fehr (19) reported cases of keratomalacia wi atrophy of the intestinal tract lining which he attributed to vitamin deficiency. Cramer et al. (20) reported atrophy of intestinal belts in expe mental animals on a vitamin A deficient diet.

Even though no actual metaplasia takes place, conditions in the dige tive tract can produce what Jolliffe (21) has called a conditioned increase requirement of vitamin A. Any condition which interferes with the absor tion of vitamin A or carotene through the intestinal wall into the bloo stream will reduce the supply of the vitamin to blood stream and bod tissues. Diarrhea, pancreatic dysfunction, and reduced bile flow are suc conditions and must be met by increase in the amounts of vitamin A i gested or injected.

Metaplasia of the Genito-urinary linings: In 1923 Wilson and DuBois (2 autopsied the body of a child that had been on a vitamin A deficient die They found keratinization of the renal pelvis. Bloch (23) and Spence (2 also noted pus formation in that region in cases of vitamin A deficienc McCullough and Dalldorf (25) have reported that sex hormones induce keratinization in rats kept on a vitamin A-deficient diet, but that this effe was not induced in rats with adequate vitamin A intake.

Mason and Wolfe (26) have definitely established that vitamin A d ficiency can produce metaplasia of the endometrium (lining of the uterus atrophy of the testes, placental injury that resulted in prolonged gestatio difficult parturition, and excessive uterine bleeding. Incidentally, the effe of vitamin A deficiency on reproduction is not to be confused with vitami E deficiency effect. In A deficiency it is the nutrition of the infant fetus tha suffers; its growth is retarded by changes in the placenta. In vitamin deficiency the female is unable to produce live young and the male test become sterile. Female rats deprived of vitamin A show an abnormal estr and a persistence of keratinized cells in the vaginal epithelium. Moore an Mack (27) have also reported lesions of the prostate gland in male rats on vitamin A-deficient diet.

Some Other Postulated Vitamin A Deficiency Effects

In the preceding sections attention has been confined to behavior of vit min A that has general acceptance today. There have been other effec postulated. But one difficulty in attributing an effect to a specific vitam deficiency lies in separation of direct action from effects that arise as consequence of inanition, growth failure etc. That is the basis for refusing credence to a therapeutic claim unless proof is supplied that the pathological or functional disturbance is definitely alleviated by dosage with the specific vitamin.

Bladder stone (Lithiasis) and Vitamin A deficiency: In 1932 Mendel (28) called attention to the frequent occurrence of bladder stone formation in his rats maintained on a low vitamin A diet. However, the American Medical Association's Council on Pharmacy and Chemistry after review of the evidence up to 1938 reported: "The existing evidence does not warrant claims for the use of any of the vitamins and especially of vitamin A in prevention or treatment of urinary lithiasis."

The Thyroid Gland and Vitamin A: Association between the thyroid gland and Vitamin A has been suggested by several observers. Rabinowitz (29) claimed that vitamin A increased iodine activity in hyperthyroidism. This viewpoint was supported by Fraser and Cameron (30) and Abelin (31) reported that animals given thyroid had an increased requirement for Vitamin A. Wendt (32) reported that thyroxine and vitamin A were antagonistic and that hyperthyroidism responded to treatment with vitamin A.

It was also noted that goats, after thyroid gland removal, gave a carotene tinted milk suggesting that thyroid played a role in the conversion of carotene to vitamin A.

The general viewpoint up to 1940–43 is illustrated by the comments of Clausen (33) and Gordon and Sevringhaus (34) which follow:

Clausen (33) stated:

> "The evidence presented appears to indicate that an antagonism exists between the action of thyroxin and that of vitamin A. It must be noted, however, that relatively large doses of both substances were used; also that few animals were used in some experiments. It seems reasonable to conclude that vitamin A may be more rapidly destroyed by an organism in which the metabolic rate is increased for any cause, including hyperthyroidism. But, it is not easy to see how vitamin A can decrease the activity of thyroxin."

Gordon and Sevringhaus (34) commented:

> "There is some evidence that an antagonism exists between the thyroid hormone and vitamin A in such a manner that experimental animals may be protected from the toxicity of desiccated thyroid feeding by large doses of vitamin A. . . . This destruction of the vitamin; however, has not been shown to be due to the thyroid effect per se, but may result from an increase in metabolism from any cause as, for example, in febrile diseases. The favorable therapeutic effects which have been reported in thyrotoxicosis from the use of vitamin A have been widely confirmed; therefore, it should not be considered in any sense as a substitute for iodine medication and surgery. In any event, a great deal more study will be required to solve the thyroid-Vitamin A relationship."

Such further study has appeared in papers reported in 1947–48. In 19 Johnson and Baumann (45) reported further evidence that thyroxine w instrumental in the conversion of carotene to vitamin A. In this paper th reported that the ability to store carotene was the same in hypo-, hype thyroid and normal animals but that hyperthyroid rats fed carotene stor more vitamin A. Also that addition of thiouracil or thiourea prevent storage of vitamin A and this effect was counteracted by administrati of thyroxine. At the time they expressed doubt as to whether this effect thyroxine could be attributed to production of increased metabolism a suggested that it was due to some other action of the gland.

Further support to the theory of the ability of thyroid to convert car tene to vitamin A came from Canadell and Valdecasas (46). In a pap before the Federation Johnson and Bauman in 1947 (47) stated that t utilization of stores of vitamin A appeared to depend primarily on t growth rate and secondarily the basal metabolic rate. Kelly and Day (4 also contributed evidence that thiouracil dosage impaired conversion carotene to vitamin A.

Another slant to the problem was given by Drill and Truant (49) wh reported that when rats on an A-deficient diet were supplemented wi carotene, ocular changes were prevented in control animals, but not thyroidectomized animals. This report raised the question of whether th roid deficiency interfered with both storage and utilization of vitamin

Taking the viewpoint that if thiouracil feeding does reduce carotene co version to vitamin A it should follow that feeding carotene to A-deficie animals previously treated with the drug would result in little or no liv storage of the vitamin, Wiese et al. (50) attempted to check this poi They reported that thiouracil treated rats did not lose all power to conve carotene to vitamin A and increase liver storage when fed as much as 34 of carotene per day. They did not check whether such conversion produc utilization of the vitamin A to prevent deficiency symptoms and indicat plans to check this point in further studies. Also they planned to determi just how much carotene must be fed daily in such cases to overcome d ficiency symptoms.

The problem of how thyroid converts carotene to vitamin A and ho thiouracil and thiourea decrease that ability is still unsettled. At prese there is evidence that raising the basal metabolism has some effect on vit min A requirement, but in their latest contribution at this writing Johns and Baumann (51) have stated:

> "Comparisons between rats of similar size suggested that metabolic rate a influenced vitamin A depletion; desiccated thyroid hastened the depletion of vitam A reserves somewhat, while thiouracil and thiourea delayed it. Quantitatively, t effect of a three-fold increase in the metabolic rate was less important for vitamin retention than a decrease in the growth rate by 50 per cent."

Vitamin A and Diabetes: In 1941 Levinson and Ratner (35) reported that carotene activates insulin and participates in cellular oxidation. They suggested that insulin treatment in diabetes be supplemented with a carotene solution. It has also been reported that in diabetes the blood content of carotene is increased and the vitamin A content lowered which suggest that the power of the liver to convert carotene to vitamin A is impaired, perhaps by reduction of insulin supply to the liver.

Lambrecht et al (36), however, suggest that the carotenemia observed in some diabetic subjects is of alimentary origin, and is not caused by inability to convert carotene to vitamin A. There was no relation shown between duration or gravity of the disease and the carotene and vitamin A content of the blood.

Mosenthal and Loughlin (37) claim that most diabetics have a plasma carotene and vitamin A content within normal limits. They found carotene above normal level in 24 percent of their cases but below normal in 11.6 percent of cases. They believe that factors other than diabetes are responsible for the regulation of the plasma carotenoid content.

Vitamin A and Tuberculosis: Getz, Hildebrandt and Finn (38) reported a study of vitamin A in tubercular and normal subjects. They first asked: "Are tubercular subjects vitamin A deficient; and secondly, is the per cent of vitamin A deficient tubercular subjects greater than the per cent of vitamin A-deficient normal persons?" As a criterion for measurement they used the dark adaptation test. In their groups 53 per cent of the tubercular subjects did show Vitamin A deficiency as against 6.6–11 per cent of the controls. They also noted that in tubercular patients the degree of vitamin A deficiency increased with severity of the disease; that tubercular subjects were benefitted by vitamin A therapy.

Conditioned Vitamin A deficiencies: It has already been noted that diarrheas, faulty absorption as in colitis and sprue and other intestinal derangements naturally interfere with the transfer of the dietary vitamins to the circulation. As a result the normal intake may still prove inadequate and require larger dosage or other methods of supplying the vitamin to the blood. A similar increase of supply is occasioned by an increase in metabolic rates, another conditioned requirement effect.

Unestablished claims for vitamin A therapy: To date there is not sufficient evidence to support claims that vitamin A is of value in combating hypertension (high blood pressure), color blindness, eye strain or rheumatic fever, or that these effects result from vitamin A deficiency. Such claims have been made.

Method of circulation of Vitamin A

Clausen (33) has summarized vitamin A behavior after ingestion as follows:

1) Vitamin A and carotene are absorbed through the intestinal wal scarcely at all through the stomach wall.

2) Absorption is preceded by fission or hydrolysis in the small intestin and it is the free vitamin (alcohol form) that is transferred across the gu wall, but esterification is immediately established with the original acid in the case of abnormally high dosage of vitamin A ester, or with acid representative of body fat under normal circumstances.

3) The passage across the gut wall is facilitated by bile and safeguarde by antioxidants while transfer to the final destination is via both lymphatic and blood stream since ligation of the thoracic duct does not interpose muc hindrance.

4) The greatest single reservoir is the liver, where the vitamin exists a a relatively high concentration of esters, eventually mobilized via the blood stream as free unesterified vitamin A.

5) The vitamin is not eliminated in the urine in normal health and t only a minor degree in fecal fat. Both vitamin A and carotene are soluble in mineral oil; carotene more soluble than the vitamin itself. Hence mineral oil dosage can definitely reduce absorption by causing its elimination in the feces.

6) Intestinal absorption of vitamin A and carotene is affected by bodily health and also by the condition of the food source.

Liver Metabolism of Vitamin A

Popper (39) has developed a technique for detecting vitamin A in tissue deposits through the induced fluorescence of the vitamin. In this technique the whole organ or a slice of tissue fixed in formaldehyde is illuminated by ultraviolet rays screened of all visible rays. If vitamin A is present, the specimen glows for a few seconds with a green fluorescence which, through the microscope, is seen to proceed from oil droplets. (Tissues containing vitamin A_2 show droplets with a reddish glow.) Using this technique they found that under normal conditions vitamin A appears to be stored in oil deposits in normal liver cells; with high intake it invades the "Kupfer" cells which then become highly fluorescent. They also found considerable storage in the kidney but none in the skin. This accounts perhaps for the long interval between therapy and healing in epidermal vitamin A deficiency.

Topical Application of Vitamin A and Carotene

Oral administration of vitamin A is the preferred method. Eddy and Howell (40) demonstrated that the shaved skin of the rat can absorb both vitamin A and carotene in amounts sufficient to produce a systemic effect. Topical application, measured by growth response, was less effective than administration of an equal amount by mouth. This suggests partial retention in the skin.

Investigation of the value of topical application of the vitamins was stimulated by reported beneficial effects of such application in treatment of skin diseases, and the topical application of cod liver oil in wound healing. [See Löhr and Unger (41) 1937.]

Detection of Vitamin A Deficiency

Getz (42) has listed the following signs of vitamin A deficiency and their time relations:

1) Conjunctival changes occurred after 13 weeks on a low vitamin A diet and this symptom practically cleared in about seven months after a return to a normal dietary intake of vitamin A.

2) Nyctalopia developed in 24 to 28 weeks of vitamin A fasting. And, normal vision was not completely restored by eleven months on a normal dietary intake of vitamin A.

3) Skin changes appeared in 46 weeks. They were reversible and cleared within one month on return to normal vitamin A intake.

4) Lowering of the plasma vitamin A content was the last symptom to appear when on a vitamin A-deficient diet.

The time of appearance of symptoms of vitamin A deficiency was also definitely affected by the extent of liver and body reserves.

The Council on Foods and Nutrition of the American Medical Association (43) has listed the following stigma suggesting vitamin A deficiency:

1) Xerosis of the conjunctivae i.e. thickening with a loss of transparency so that only the more superficial vessels of the bulbar conjunctiva are clearly seen, associated with more or less yellow pigmentation, especially along the horizontal meridian of the eye ball; infrequently associated with small foamlike plaques called Bitot's spots.

2) Follicular conjunctivitis indicated by hypertrophy of the follicles, particularly of the lower eye lids.

3) Kerato-malacia or softening of the cornea. A thickening with subsequent ulceration and necrosis of the cornea present only in the most severe and advanced Vitamin A deficiency.

4) Nyctalopia or night blindness detectable by measurement of dark adaptation and conspicuous only in cases of advanced and severe vitamin A deficiency.

Skin Changes

1) Papular eruptions of pilosebaceous follicles. A grater like peel which in the early stages resembles goose flesh, but when more fully developed presents the picture of keratosis pilaris (broad conical elevations investing

the hair follicles). The extensor surfaces of the arms and thighs and th flexor surfaces of the legs are primarily affected.

2) Xerosis or steatosis of the skin. A dryness, scaliness and crinkling o the skin, in extreme cases resembling alligator skin. In the early stages th condition is associated with keratosis pilaris but it persists and extend after the follicles have disappeared, the body hairs being broken and late lost. All parts of the body are involved but the skin of the extremities particularly of the legs, is more severely affected than the skin of head an trunk.

Kruse (44) recommended for the early detection of vitamin A deficienc a bio-microscopic ocular examination which will show xerosis of the conjunctivae. He claims that this condition precedes nyctalopia as an earl sign of deficiency.

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Section II. The Chemical Nature of Vitamin A

The form of vitamin A designated by McCollum (1) as "unidentified dietary factor, fat-soluble A" was isolated from the nonsaponifiable fraction of certain animal fats. Fish liver oils were early found to be rich natural sources of this vitamin.

When plant materials were bioassayed for vitamin A potency it was found that yellow vegetables contained more than white vegetables. Yellow corn and carrots proved rich sources; white corn and parsnips contained decidedly less vitamin. A similar contrast appeared between white potatoes and sweet potatoes.

In 1919 and 1920 Steenbock (2) suggested that the vitamin A in yellow corn was related to the yellow pigment carotene. Later investigators found that this pigment was a "provitamin A", convertible in the animal body into the nearly colorless vitamin A. The richness of green leaves in vitamin A was also explained when it was shown that in such leaves the green pigment, chlorophyll, masked a high content of this yellow carotene pigment and that chlorophyll-rich leaves always showed this high content of carotene.

The conversion of carotene to vitamin A in animals was first demonstrated by Moore (3) who showed that when rats were fed carotene thei livers stored vitamin A instead of carotene. This conversion of carotene t vitamin A was first postulated to occur in the liver through the action of hypothetical enzyme called carotinase. Sexton et al. (4) have reached different conclusion. They claim that carotene injected into the rat doe not change into vitamin A; that the conversion of ingested carotene t vitamin A probably takes place while passing through the intestinal wall This view appears well established by the work of Deuel and others (23 (24) (25).

Ability to make the conversion varies with the animal species. For example, the Holstein variety of cow appears to have a very efficient conversion ability as a result of which its milk is white. The Jersey and Guernsey cow on the other hand transmit considerable carotene unchanged into their milk with a resultant increase in yellow color. This appears confirmed by the fact that when the butters of the two kinds of milk are assayed for vitamin A potency they show little difference if the cows are on similar carotene intake

Herbivorous animals appear able to secure their entire need of vitamin A by eating carotene. Human beings, according to Sherman et al. (5), apparently need to ingest twice as much carotene as vitamin A in order to meet their daily vitamin A needs.

Forms of carotene

Wachenroder (6) isolated carotene, called by him carotin, from carrots as far back as 1826, long before it was assigned any physiological significance. Study of crude carotene extracts showed its existence in at least 4 forms convertible to vitamin A. These four forms were named alpha, beta, and gamma carotene and cryptoxanthin. Of these, beta-carotene was most completely convertible to vitamin A and today it is generally accepted that .0006 mg. of beta carotene is equivalent to one U. S. P. unit of vitamin A. (For forms of carotene and their relative activity see Table 2.)

As previously stated the permanent Commission on Biological Standardization of the League of Nations officially defined the International Unit of Vitamin A as the biological activity of 0.6 micrograms of beta-carotene. This has been the basis of saying that beta-carotene contains 1,666,667 units of vitamin A per gram.

The U.S.P. unit of vitamin A allegedly had the same biological value as the International unit but when assayed by reference to the U.S.P. Reference cod liver oils poor correlation was obtained.

Since January 1st 1948 a new reference standard has been used. This is a solution of crystalline vitamin A acetate in cotton seed oil contained in a special gelatin capsule. This solution contains 3.44 mg of vitamin A acetate in each gram. The solution has a biological potency of 10000 U.S.P. units of vitamin A per gram. Each capsule contains 250 mg (±1 mg) of the oil solution thus having a Vitamin A potency of 2500 units per capsule or an amount of crystalline vitamin A acetate equivalent to 0.75 mg of vitamin A alcohol per capsule.

This oil used in the capsules has been carefully assayed biologically against solution of the International Standard and was shown to provide almost exactly 10000 International units of vitamin A per gram. Since 3.0 mg of vitamin A is equivalent to 10000 U.S.P. units this gives an official definition that one International unit equals one U.S.P. unit equals 0.3 micrograms of vitamin A.

Karrer and coworkers (7) first elucidated the structure of beta-carotene and showed that it consisted of two beta-ionone groups connected by a straight CH chain link. (See figure 2.) According to Karrer, vitamin A is formed by breaking this chain in the middle to form two molecules of vitamin A alcohol. The other forms contain only one beta-ionone group and could produce only one molecule of vitamin A. (See figure 3.) Just how this split is accomplished is still controversial. This split could be produced by hydrolysis but there is some evidence today (Clausen (8)) that oxidation is involved. At any rate, in estimating vitamin A potency of a source from its carotene content it is important to determine the amounts and kinds of carotene present. According to Embree (5): "It is not yet certain that some of the carotenoids are not themselves directly used by certain animals and can thus be classified as vitamins, not provitamins."

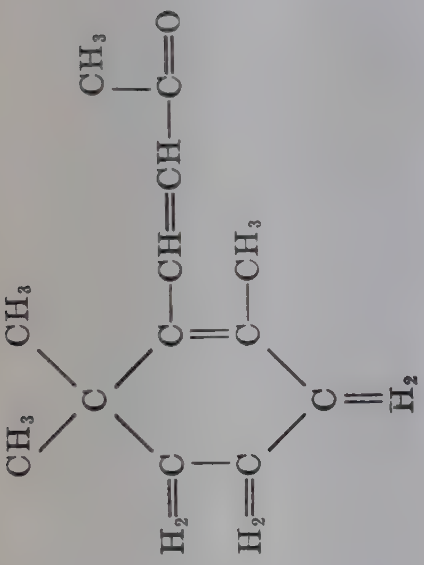
With (9) points out the comparatively high activity of beta-carotene for hens and the unusually high activity of cryptoxanthin for this species. He suggests that cryptoxanthin is itself a vitamin for the hen.

Table 2 indicates that these four forms have now been shown to yield certain other isomers. In Table 3 Embree (5) also gives the absorption spectra for detection of pure carotene (beta) content.

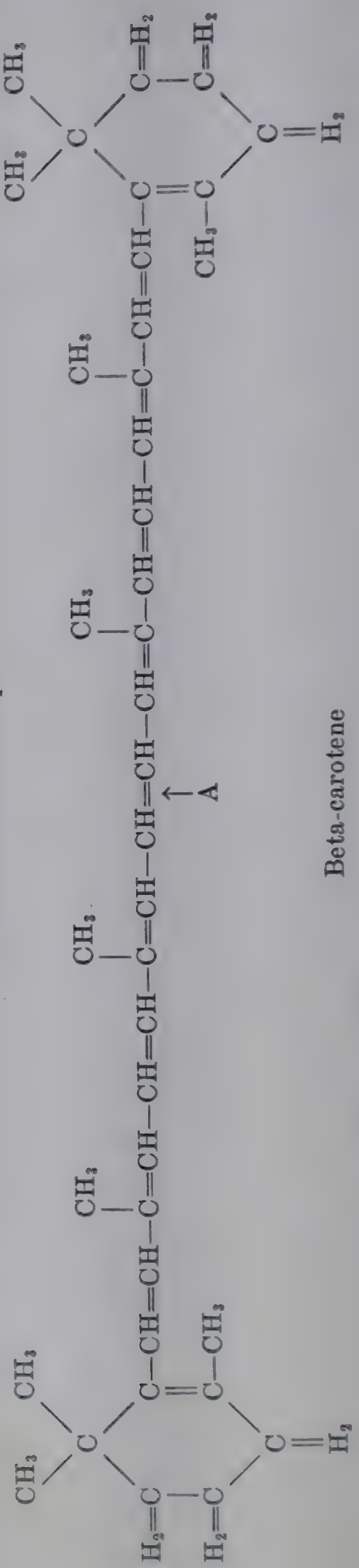
Hunter and Williams (10) claimed to be the first to convert carotene to vitamin A by chemical means. They split the beta carotene molecule with hydrogen peroxide to form vitamin A aldehyde and subsequently reduced the aldehyde to the vitamin A alcohol.

Forms of Vitamin A

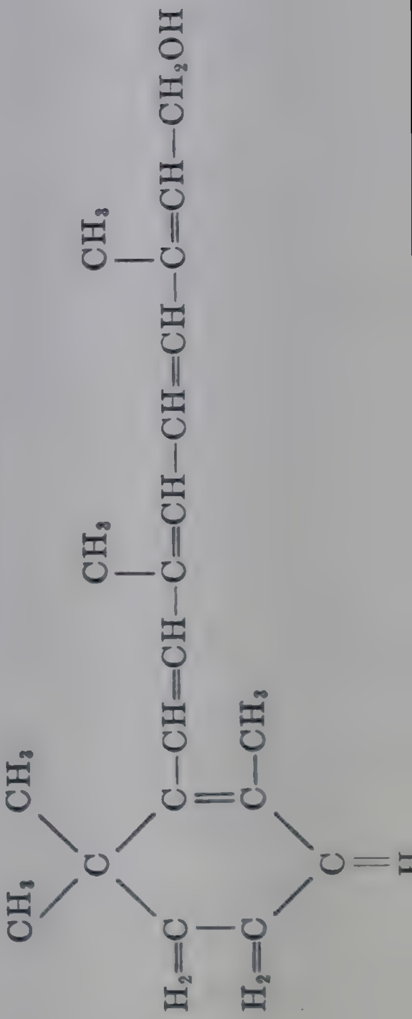
Like carotene, vitamin A itself occurs in different forms. Free vitamin A is an alcohol, which means that it can form esters. Hickman (11) reported the recovery of such esters by molecular distillation of fish liver oils. He claimed that the vitamin probably occurs in the liver oils as an ester and that the esters are better absorbed from the gut than the alcohol form. English workers have questioned this latter claim. Mead, Underhill and

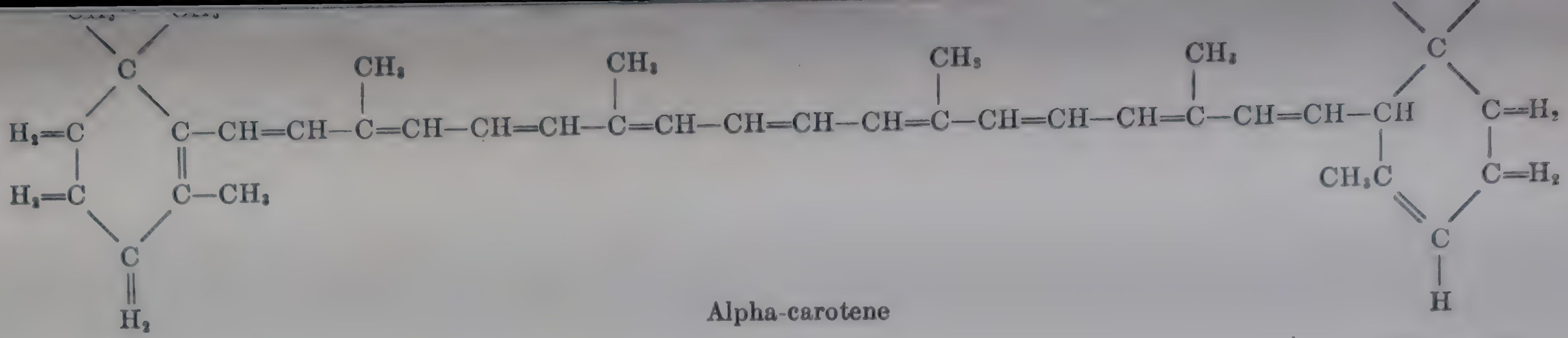


Beta-ionone Group

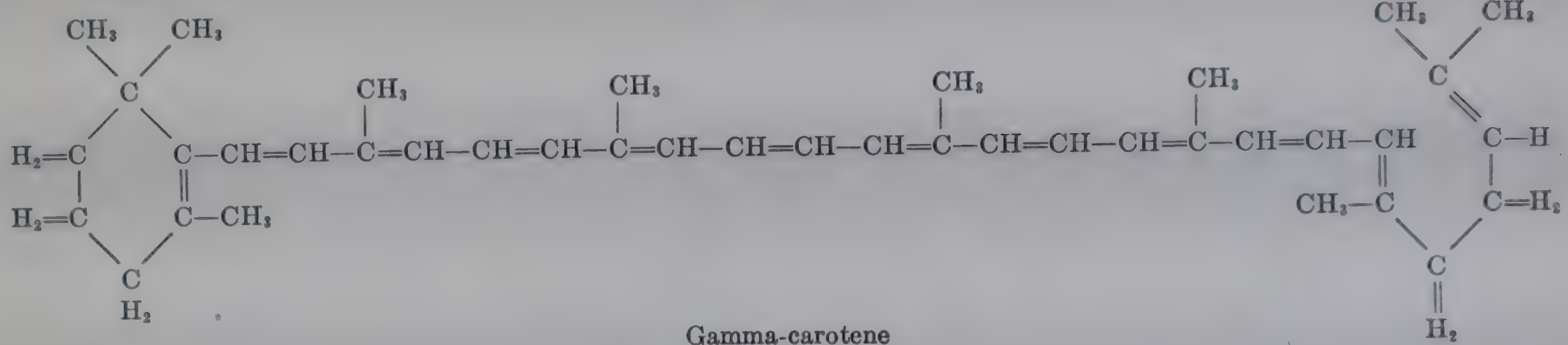


Beta-carotene

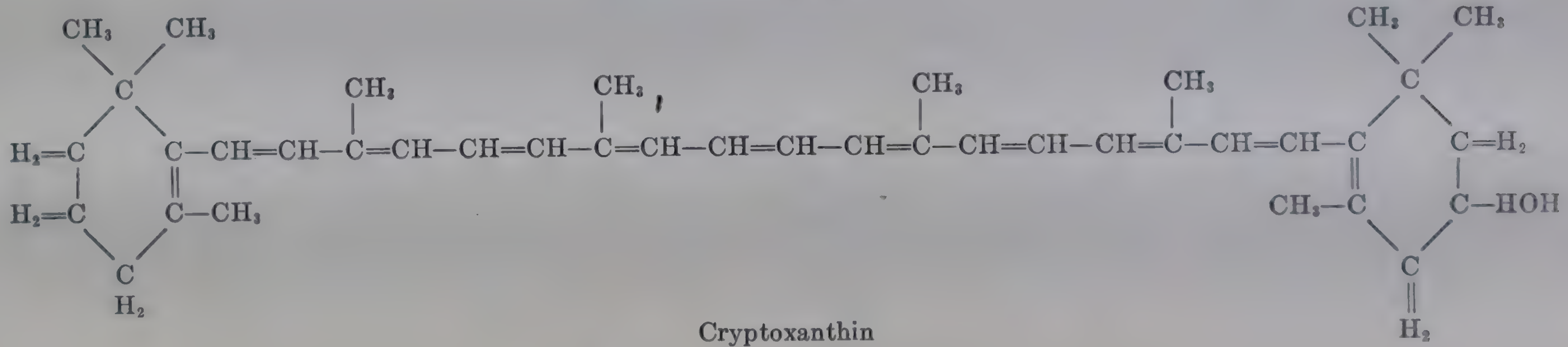




Alpha-carotene



Gamma-carotene



Cryptoxanthin

FIG. 3. FORMS OF PRO-VITAMIN A

Coward (12) hold that for a corresponding A content the biological potenc of the aliphatic esters is the same as that of the alcohol form. Baxter, Robe son and Harris (13) give the comparison in activity of the vitamin A ester as shown in Table 4.

Vitamin A_1 and A_2: Heilbron (14) reported that the blue solution obtaine

TABLE 2

Biological activities of carotene for the rat

(After Embree, N. D.: Ann. Rev. Biochem., p. 333 (1947))

Type of carotene	Relative activity	References
All trans beta carotene	100	Deuel, H. J. et al.
Neo-beta-carotene U	38	Arch. Biochem., 6: 157 (1945)
Neo-beta carotene B	53	Ibid., 7: 247 (1945)
All trans alpha-carotene	53	Ibid., 6: 157 (1945)
Neo-alpha-carotene U	13	Ibid., 6: 157 (1945)
Neo-alpha-carotene B	16	Ibid., 7: 247 (1945)
All trans gamma-carotene	28	Ibid., 5: 365 (1944)
Pro-gamma-carotene	44	Ibid., 5: 365 (1944)
Cryptoxanthin	57	Ibid., 7: 447 (1945)
Neo-cryptoxanthin A	42	Ibid., 10: 491 (1946)

TABLE 3

New preparation of beta carotene

(Devine, J., Hunter, R. F., and Williams, N. E.: Biochem. J., 39: 5 (1945)

Solvent	Wave length of main band in $\mu\mu$	$E^{1\%}_{1\,\text{cm.}}$	Previous literature
Cyclohexane	456	2490	2400 @ 456 $\mu\mu$
Chloroform	465	2370	2200 @ 463 $\mu\mu$ 2310 @ 463 $\mu\mu$
Hexane	453	2610	2580 @ 450 $\mu\mu$ 2540 @ 450 $\mu\mu$ in petrol 2500 @ 450 $\mu\mu$ in petrol
Benzene	465	2370	

by heating fish liver oils with antimony trichloride frequently exhibited a low intensity band at 695 $\mu\mu$ in addition to the usual one at 620 $\mu\mu$. When certain liver oils of fresh water fish were so assayed the blue antimony solutions produced bands at both 620 $\mu\mu$ and 695 $\mu\mu$ but greater intensity at 695 $\mu\mu$. The oils themselves showed a maximum absorption at 345–350 $\mu\mu$ instead of the 328 $\mu\mu$ shown by marine fish liver oils.

These observations preceded the isolation of what is now called Vitamin A_2 from the fresh water fish liver oils. The term, vitamin A_1 was retained for designation of the vitamin found in the marine fish liver oils. As already noted, vitamin A_2 forms a different rod pigment (porphyropsin) from vitamin A_1 (rhodopsin). (See Section I and figure 1.) See also Table 1.

Vitamin A_2 functions in the same way as vitamin A_1 to control visual acuity. Jensen et al. (15) reported that high dosage of vitamin A_2 was toxic for rats, but appreciable biological activity was noted at dosage levels ordinarily used in biological assays. Schantz et al. (16) experimented to see whether A_2 could be substituted for A_1 in the tissues of the rat. Vitamin A-depleted rats were fed 100 units of vitamin A_2 per day. After twelve weeks of feeding substantially all the vitamin A in the blood was A_2, and the visual pigment was porphyropsin instead of rhodopsin. However, the rats were somewhat below normal in growth and reproduction compared with rats on 100 units of A_1 per day. Shantz (26) has announced the separation of the pure vitamin A_2 from pike livers. The ester had a melting point of 76–77° and $E^{1\%}_{1\,\mathrm{cm}}$ of 1190 at 341$\mu\mu$. The pure A_2 and two absorption maxima at 351$\mu\mu$ E equalled 1460; at 287$\mu\mu$ E equalled 820. With antimony trichloride there was an absorption maximum at 693$\mu\mu$ and an E of 4100. Their biological tests on A_2 gave a value of 1,300,000 I.U. per gram.

TABLE 4

Properties of vitamin A forms

(After Baxter, J. G., Robeson, C. D. and Harris, P. L.: Ann. Rev. Biochem., 12: 354, (1943)

Forms of vitamin A	Crystal structure	M.P.	Potency of content
		°C.	*units per gram*
Alcohol	Yellow prisms	64	4,000,000
Acetate	Primrose yellow prisms	57–58	4,040,000
Palmitate	Pale yellow plates	27–28	4,230,000
dl-Succinate	Branching prisms	76–77	3,590,000

Kitol: In 1943 Embree and Schantz (17) reported the extraction from whale oil of a compound which they called "kitol". It was a pale yellow oil at above room temperature, a solid glass at 25°C and a probable chemical formula of $C_{40}H_{58}(OH)_2$. Its vitamin A biological activity was low (much less than 140,000 units per gram). On heating, however, it acquired activity and an absorption band identical with vitamin A_1 at 328 $\mu\mu$. It is therefore a true provitamin A, but its significance in whale metabolism has not been explained.

Cyclized Vitamin A: A substance that occurs in traces in all vitamin A bearing oils and which is produced whenever vitamin A is "mistreated" has been called cyclized or anhydro-vitamin A [Heilbron (18)]. Its creation is a process of simple dehydration and it is not biologically active.

Neo-vitamin A: Robeson and Baxter (19) have reported a new form of vitamin A which they call neo-vitamin A. It appears to be a geometrical isomer of vitamin A and they claim that most liver oils contain a mixture of the two forms in the proportion of 2 parts of vitamin A and 1 part of neovitamin A. The two forms are apparently equal in biological activity.

Synthetic Vitamin A: Progress has been made in the synthesis of vitamin A, and some has been marketed at this writing. Milas (20) has reviewed the progress to date and Embree (5, 21) has also published a similar review of experimental work in this field. Hanze et al. (22) have reported the successful synthesis of a vitamin A methyl ether which proved identical with vitamin A in absorption spectra and biological activity.

TABLE 5

Properties of other vitamin A forms

I. Vitamin A alcohol ($C_{20}H_{29}OH$)
    Melting point: 63–64 deg.
    Maximum absorption @ 325 $\mu\mu$
    Potency in I.U. per gram: $3\text{–}3.3 \times 10^{-6}$
    $E_{1\,\text{cm.}}^{1\%}$ 1750
II. Vitamin A acetate
    Melting point: 57–58 deg.
    $E_{1\,\text{cm.}}^{1\%}$ 2000
III. Neo-vitamin A
    Melting point: 59–60 deg.
    Maximum absorption 328 $\mu\mu$
    $E_{1\,\text{cm.}}^{1\%}$ 1675
IV. Beta carotene ($C_{20}H_{46}$)
    Melting point: 187 deg.
    Maximum absorption: 521–485 $\mu\mu$
    Potency: 1.66×10^{-6}

Chemical and Physical properties of vitamin A forms: Tables 2 and 5 relate some of the properties of the various forms of vitamin A-potent materials. These properties serve for identification of the types, but for determination of physiological activity the bioassay method in a particular species becomes essential. Both vitamin A and carotene can be destroyed by oxidatio and by exposure to light. Vitamin A is more stable than carotene. Neithe are affected by heat alone, but in the presence of oxygen, heat accelerates the destruction. In capsule and tablet offerings of the vitamins, wheat germ oil containing vitamin E is often used as an antioxidant to prevent destruction.

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SECTION III. EVALUATION OF VITAMIN A FORMS FOR HUMAN AND ANIMAL USE

In Section I are outlined the physiological effects to be expected in vitamin A deficiency and the conditions requiring vitamin A therapy. It is evident from the data in Section II that chemical identification of a form of vitamin A or carotenoid in a source and its content fails to determine its actual physiological value in any given species. Such evaluation demands study of the behavior of these forms in meeting the requirements of human beings and specific animal species. We are especially interested in behavior in cattle, swine, poultry, sheep, and other domestic animals.

Because the rat behaves quite similarly to human beings, its response to deficiency and therapy has been extensively used to evaluate values for human beings. The analogy is not perfect and additional tests on human subjects have been necessary to get more exact values.

Blood content measurements, determination of amounts necessary to maintain normal dark adaptation, effect on liver storage and amounts to correct visible evidences of vitamin A deficiency have all been used to reach conclusions as to human and animal actual requirements.

Human Requirements of Vitamin A

It has become customary to express quantitative human needs of vitamin A in U. S. Pharmacopoeia units (1) (equivalent to International units). In prescribing label claims for vitamin A values the Food and Drug Division of the Federal Security Administration (2) set the minimum daily adult allowance at 4000 units; this the minimum amount necessary to prevent visible symptoms of vitamin A deficiency. Also it is required that in a label statement of vitamin A potency there must be recited what fraction of this requirement is supplied by a given unit or quantity (capsule, tablet, etc.) of the offered product. The object of such statement is two-fold: first, to protect the consumer against false claims, and second, to provide a basis for check by Food and Drug Inspection.

TABLE 6

Suggested daily allowances of vitamin A

(After the Food and Nutrition Board of the National Research Council, 1948)

Subjects	Calories	Units of vitamin A	Children of both sexes up to 12 yrs.	Calories	Vitamin A units
Man (154 lbs.)	2400–4500	5000	Under 1 yr.	100/2.2 lbs.	1500
Woman (123 lbs.)	2000–3000	5000	1–3 yrs. (29 lbs.)	1200	2000
Woman (latter half of pregnancy)	2400	6000	4–6 yrs. (42 lbs.)	1600	2500
Woman (during lactation)	3000	8000	7–9 yrs. (55 lbs.)	2000	3500
			10–12 yrs. (75 lbs.)	2500	4500
Girls					
13–15 yrs. (108 lbs.)	2600	5000			
16–20 yrs. (119 lbs.)	2400	5000			
Boys					
13–15 yrs. (103 lbs.)	3200	5000			
16–20 yrs. (141 lbs.)	3800	6000			

During the war years the Food and Nutrition Board of the National Research Council (3) made a special study of human needs and formulated suggested unit daily allowances (not requirements) which were first published in 1941 and revised in 1945 and 1948. See Table 6. In ascertaining the suggested allowances the Board utilized the measurement of dark adaptation in human beings; by determination of the amount necessary to maintain normal dark adaptation with addition of a factor of safety to cover variations in absorption and utilization of the vitamin.

The Council of Pharmacy and Chemistry of the American Medical Association (4) has also drafted rules for therapeutic use of vitamin A: 25000 units twice daily for two months or more for general treatment and 25000 units three times daily for prolonged or chronic deficiency. They have also stated that they found no need for giving more than 25000 units per dose.

The U. S. Pharmacopoeia sets the average daily dose of cod liver oil for infants and adults at 8 cubic centimeters or 2 fluid drachms, i.e. approximately 6800 units of vitamin A daily.

There have, however, been other estimates of human needs for vitamin A. Batchelder and Ebbs (5) put the requirement at 37–40 units per pound of body weight per day; for an adult weighing 150 lbs. Nylund (6) gives a lower value: 12.5–20 units daily per pound of body weight. A preliminary report of the British Ministry of Foods, based on a study of 23 human cases over a period of 2 years, concludes that 2500 units of vitamin A or 5000 units of beta-carotene daily are adequate for human adult maintenance with a fair margin of safety.

Sherman et al. (7), however, pointed out that as a factor for producing longevity the daily allowance of vitamin A may well be increased considerably above the allowances of the Research Council. He has suggested amounts 2 to 4 times such allowances, i.e., 10000 to 20000 units daily.

Blood content as a basis for estimating requirements

From the clinicians viewpoint a correlation between blood content of vitamin A and requirement would be highly desirable. Unfortunately deficiency effects can occur before there is significant change in blood content.

According to Clausen (8) there is definite tendency to maintain a constant plasma concentration of vitamin A. Under fasting conditions the vitamin A in human plasma is present as free alcohol but when large amounts are ingested the increased A appears in the ester form. It is possible to measure accurately the vitamin A or carotene content of plasma in small amounts of blood.

Such measurements have been made. Gordon and Sevringhaus (9) put the normal blood value at 88–100 units of vitamin A per 100 ml. of plasma and consider that values below 60 indicate a deficiency.

Popper and Steigman (10) have reviewed the clinical significance of plasma vitamin A values. In this review they aimed to answer these questions: What determines plasma level and how may knowledge of plasma level be of value to the clinician? Their conclusions may be summarized as follows:

First, many months may elapse after withdrawal of vitamin A from th diet before plasma content decreases. The plasma content is to a degre controlled by the amount of storage in the liver. After dietary supply ceases the liver stores may serve for a considerable time to maintain normal bloo content. At present, there is no means of measuring liver content in th human subject. In brief, assay of plasma content of either vitamin A o carotene is of little value in estimating inadequacy of dietary intake.

A rapid drop in plasma content may have diagnostic value however It may help to indicate disturbances in absorption which occur in celia disease, sprue, colitis, pyloric obstruction, pancreatic fibrosis, congenita atresia of the bile ducts, intestinal obstruction, severe pulmonary tuberculosis, infantile eczema and liver disease.

A drop may occur with increase of requirements in pregnancy and lactation, in hyperthyroidism and febrile diseases or infection. In the treatment of these diseases the plasma values help the physician to follow the success of treatment.

Lack of adequate vitamin E in the diet or of lipoid carriers in the blood also results in drop of plasma A content.

Relation of Human Requirement to Forms of Vitamin A

So long as vitamin A alcohol or ester is used to supply the need, demonstration of the unit values in a given source truly indicates its value as a dietary supply. Today, however, human beings rely for their daily supply of the vitamin on many foods in which the vitamin is in the form of carotene, not vitamin A. Group I of the Basic Daily Seven foods was suggested for that reason (see Table 55).

When sources are assayed chemically or spectrometrically for beta-carotene it is assumed that each .0006 mg. of beta-carotene is equivalent to one unit of Vitamin A. But it has been proven that alpha and gamma carotenes have only about half the biological value of beta-carotene and that human beings do not utilize carotene as efficiently as they do vitamin A. Hence one should take at least twice as many units of beta-carotene or 4 times as many units of alpha and gamma carotene to get the equivalent of one unit of vitamin A alcohol or ester.

It has already been mentioned that utilization of both vitamin A and carotene is influenced by the vitamin E content of the diet. (For details of this action see Chapter IV.) W. C. Sherman (14) has also produced evidence by means of rat experimentation to show that the yellow plant pigments known as xanthophylls which usually accompany carotene in natural sources may function in a manner similar to vitamin E.

In estimating the value of a food source it is also necessary to take into account its digestibility, the ability of the subject to extract the vitamins from the food in the process of digestion. The form of the carrier influences this extractibility; carotene in oil or in a fat such as margarine or butter is better utilized than carotene in vegetables. Also the condition of the vegetable when eaten is a factor. Raw carrots, for example are said to yield only one per cent of their carotene but cooking increases this yield to 35 per cent.

Excess of Vitamin A

It has generally been held that an amount of vitamin A above requirement exerts no toxic effect. However there appears to be an amount that can produce toxicity. This was discovered in a study of what is called telangiectasis.

Telangiectasis in cattle livers has been noted and described as far back as 1900. The abnormality is characterized by dark red or purplish depressed areas beneath the capsule of the liver. They may vary in size from a few to 15 millimeters in diameter. Only one lobe may be involved. The dark areas are spongy and usually engorged with blood. Herbst, Pavcek and Elvehjem (15) made a study of these livers and their effect when fed to test animals. The vitamin A content proved to be abnormally high; as much as 132000 units per 100 grams as compared with an average normal liver content of 50000. Their content of carotene was *not* above normal. The iron content was also high, 50 per cent above normal.

When these abnormal "high-vitamin A livers" were fed to rats toxic effects ensued; growth failure, paralysis and spontaneous bone fractures. As fed they supplied as high as 15000 units of vitamin A per day, an enormous increase over the rats daily requirement (about 6 units per gram of food intake). Feeding vitamin A alone in similar amount duplicated the toxic effect.

There is then a toxic limit to vitamin A dosage, but little danger in the amounts usually consumed even in therapeutic treatment of vitamin A deficiency.

Excess of carotene in the blood can produce a yellow pigmentation of the skin but such carotenemia is without toxic effect on the subject.

Vitamin A and Domestic Animals

In addition to the Reports of the Food and Nutrition Board of the National Research Council on human allowances, the Committee on Animal Nutrition also reported allowances for domestic animals (16). Manifestation of vitamin A deficiency in such animals varies somewhat from the effects on human beings.

In cattle the first easily detected symptom of vitamin A deficiency is night blindness. The next conspicuous symptom is muscular incoordination; staggering gait and perhaps convulsions. The seizures are caused b elevation of the cerebrospinal pressure. Papillary edema may also resul and other localized paralyses may occur. Instead of xerophthalmia in the co there is excessive tear formation (lacrimation). The cornea of the ey may become keratinized and if infected develop ulceration. In dairy cattl milk formation increases the requirement over the minimum necessary fo growth or weight maintenance. Similar results are produced in sheep, an lack of vitamin A results in inability to reproduce normally. In swine there is lameness due to muscular incoordination, night and day blindnes and sometimes diarrhea. Symptoms in swine are slow in developing. Blindness in new-born pigs can result from inadequacy of maternal diet.

Poultry on a severely vitamin A deficient diet will show symptoms a early as in three weeks. Growth is retarded, chicks show general weakness emaciation and a staggering gait and ruffled plumage. Resistance to infection is lowered. The secretions of the intestinal mucous glands, tear glands and salivary glands fail. Opaque keratinization of the third eye lid may b observed, and with infection a sticky fluid is formed which may cause th eyelids to stick together. Pathological lesions in poultry, observed o autopsy, are confined mainly to the mouth, pharynx, esophagus, respiratory and urinary systems. In the mature fowl symptoms of vitamin A deficiency may develop more slowly, but the eye affection becomes mor acute. A cheesy exudate from the eyes and a sticky discharge from th nostrils occurs. Egg production and egg hatchability are markedly reduced The symptoms of turkey poults are in general similar to those of hens an chickens but are usually more acute.

Carotene is the main source of vitamin A for herbivorous animals an poultry which they handle efficiently. The actual requirements of eac type of domestic animal have been worked out by the Committees o Animal Nutrition and summarized in bulletins (16) to which the reader i referred for details.

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CHAPTER III. VITAMIN D

Section I. Functions of Vitamin D

The second fat-soluble vitamin to be identified is called vitamin D. Its discovery followed a search for a preventive and cure of rickets.

In 1918 Mellanby (1) had shown that cod liver oil was an effective agent for producing hardening of bones in puppies. It was already established that cod liver oil was rich in vitamin A and at first it was suggested that vitamin A was the responsible vitamin.

Cod liver oil therapy as a preventive of rickets was not a new treatment in 1918. Schuette in 1824 was the first to use it for that purpose, and years before the discovery of vitamins Jacoby at the Columbia University Medical School used to tell his students that in the treatment of rickets: "If you can't get them out of doors, give them cod liver oil."

That vitamin A in cod liver oil was not the effective vitamin was proven by Hess and Unger (2) in 1920. They showed that infants taking plenty of vitamin A in the form of milk could still contract rickets.

In 1921 McCollum et al. (3) bubbled air through heated cod liver oil and thus destroyed its vitamin A content, but the treated cod liver oil still retained its antirachitic powers. From this result they postulated the existence of another fat-soluble, heat stable vitamin in the oil to which they assigned the name vitamin D. The actual isolation and chemical identification of the vitamin came later.

The Nature of Rickets

The medical dictionary defines rickets as follows:

> "A constitutional disease of infancy characterized by impaired nutrition and changes in the bones, the symptoms being a diffuse soreness of the body, a slight fever, and profuse sweating about the head and neck, and changes in the osseous system consisting of a thickening of the epiphyseal cartilages and periosteum and a softening of the bones."

Park (4) has described the pathology of rickets as follows:

> "The pathological changes depend on and hence follow a change in the chemical structure of the blood. Most commonly the inorganic phosphate has fallen to a subnormal concentration, less often calcium; occasionally both. The result is a depression of the solubility product (S.P.) which governs the precipitation of calcium phosphate from the blood (lymph) into the cartilage and bone. When the S.P. falls below a certain critical value, lime deposition in bone and cartilage becomes irregular and, if the value is low enough, deposition stops altogether. The failure in lime salt deposition is responsible for the weakening of the bones and at the same time is the cause of almost, if not all, the histological changes. The first demonstrable pathological change in

rickets is the failure of lime deposition in the proliferative cartilage of the epiphysis and in the newly forming bone."

Solubility Product

The solubility product referred to by Park and used in the diagnosis of rickets by Howland and Kramer (5) is the product of the concentrations of calcium and phosphorus in the blood. Howland and Kramer suggested that if the product is less than 40, due to either low phosphorus or low calcium in the blood, rickets will occur. That healing begins when the product reaches 40.

TABLE 7

Distribution of phosphorus in blood

I. After Shohl (6)

In blood	Total phosphorus	Lipid phosphorus	Ester phosphorus	Inorganic phosphorus
	mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*
Whole blood	38	12	23	3
Serum	68	7	50	?
Blood cells	13	8	1	3–4

II. After Bodansky (7)

Form	Range	Average
Inorganic PO_4 in serum	2.5–5.5 mg./100 ml.	3.5 mg./100 ml.
Lipoid phosphorus in plasma	5–12 mg./100 ml.	7.5 mg./100 ml.
Organic phosphorus in corpuscles	40–75 mg./100 ml.	53 mg./100 ml.

N.B. The main difference between infants and adults is higher content of inorganic phosphate in the infant blood; 5.0 ± 1 mg./100 ml. in infants, 3.0 ± 1 mg./100 ml. in adults. The average calcium is normally 10.0–10.5 mg./100 ml. wholly in the serum; slightly higher in infancy and in the last months of pregnancy; slightly lower in extreme old age.

In the blood and lymph both calcium and phosphorus are present, the latter in both organic and inorganic combination. In figuring the solubility product the phosphorus present in inorganic form is used. (For distribution of calcium and phosphorus in blood see Table 7.)

The average calcium/phosphate ratio in normal individuals is not far from 1.2/1. With adequate amounts of calcium and phosphorus in the diet a ratio in the diet of 1/1 will maintain a normal blood content and a satisfactory solubility product without addition of vitamin D. But if the diet ratio is unsatisfactory, use of vitamin D will correct the faulty ratio and maintain normal blood content.

Bone Composition

Bone is known to be mainly a mineral substance corresponding t natural mineral known as dahlite. Its chemical formula may be writ $n(Ca_3(PO_4)_2{:}Ca\ X)$ in which "X" may be CO_3, OH, or F, and "n" m be 2 or 3. In human bone "X" is mainly carbonate (CO_3) and the mine part of the bone is a mixture of calcium phosphate and calcium carbon in the ratio of two molecules of the former to one of the latter; in percent about 88 per cent calcium phosphate and 12 per cent calcium carbona

The ability of these salts to precipitate out of a solution and deposit growing bone depends upon the product of the calcium and phosphate i in the solution. McLean and Hastings (8) state that if the ion prod ($Ca^{++} \times PO_4^{---}$) is less than 10^{-27} calcium phosphate will go into a stay in solution; that to initiate precipitation or deposition in cartil the product must not exceed $10^{-23.5}$, but that if once started, precipitat and deposition will continue until an ion product of 10^{-27} is reached.

It is well known that new bones are mainly cartilage or gristle; that t is stiffened by deposition of lime salts, and if not so stiffened become eas bent, producing bow legs and other deformities.

It should be evident from the preceding statements that in preventi rickets the task is to secure proper proportion of calcium and phospho in the blood and a satisfactory concentration of each plus a protective d age of vitamin D.

Rachitogenic Diets

If the diet does not contain sufficient amounts of calcium and phospho or in proper proportions rickets will occur. This was first demonstrated Sherman and Pappenheimer (9) in 1921. They built two diets for rats, one of which rickets resulted, on the other rickets was prevented. The di differed mainly in the calcium/phosphorus ratio of their content. La McCollum (10) and Steenbock and Black (11) used similar principles develop rachitogenic diets. The Steenbock-Black diet is employed today create rickets in test rats used to establish unitage of vitamin D sourc Addition of vitamin D to such diets produces healing of rachitic bones a such biological tests form the basis for expressing U. S. Pharmacopo (12) unitage. The composition of these diets is shown in Table 8.

Factors controlling the Solubility Product

Shohl (6) discusses two kinds of rickets; one (most common) when t serum phosphate is low and the serum calcium higher. Vitamin D is t only known vitamin curative of this type of rickets. A second type resu when the calcium of the serum is low and the phosphate high. Shohl h also pointed out that even if the diet supplies both calcium and phosph in adequate amounts and proper proportions, conditions can arise to render the salts insoluble in the digestive tract and hence un-absorbable into the blood. The presence of oxalic acid in spinach, rhubarb and some other food stuffs can produce precipitation of insoluble calcium oxalate. Its presence in the digestive tract can not only render the calcium of the vegetables insoluble but it can also have a similar effect on any other food calcium present. That is why one finds in tables giving the calcium content of vegetables a question mark after those that contain oxalic acid. The amount available will be the amount of calcium in excess of that precipitable by the amount of oxalic acid in the swallowed portion.

TABLE 8

Rachitogenic diets for rats

I. Sherman and Pappenheimer (9)

Ingredients	Diet 84, rachitogenic	Diet 85, anti-rachitogenic
Patent flour	95%	95%
Calcium lactate	2.9%	2.5%
Sodium chloride	2.0%	2.0%
Iron citrate	0.1%	0.1%
Potassium phosphate	0.0%	0.4%
Ca/P ratio	6.5/1	3/1

II. McCollum (10)

Ingredients	Diet 3143, rachitogenic
Whole wheat	33%
Whole yellow corn	33%
Wheat gluten	15%
Gelatin powder	15%
Calcium carbonate	3%
Sodium chloride	1%
Ca/P ratio	4/1

III. Steenbock and Black (11)

Ingredients	Diet 2695, rachitogenic
Yellow corn	76%
Wheat gluten	20%
Calcium carbonate	5%
Sodium chloride	1%
Ca/P ratio	4/1

Shohl also cites the following elements which render phosphates insoluble and unabsorbable: beryllium, magnesium, strontium, iron, lead and thallium.

Solubility and availability of food calcium salts are also affected by the acid/base balance of the body. Preponderance of acidity favors calcium salt solubility.

Hamilton and Schwartz (13) converted normal diets into rachitogenic diets by adding alkalies plus acidic ash; rachitogenic diets into normal diets by addition of organic acids and alkaline ash. For the first effect they used ammonium carbonate and ammonium chloride. For the latter effect they used the following salts and acids listed in the order of their effectiveness: sodium acetate, sodium tartrate, sodium bitartrate salts, citric and tartaric acids. Shohl comments on these experiments that the action of the organic acids was not due solely to their hydrogen ion contribution, but that the acid ions themselves play a role and that the citrate ion is more effective than the tartrate ion. Hurni (32) explains the action of citrate as a result of the formation in the digestive tract of a non-ionized calcium citrate complex. The absorption of calcium from the gut is unaltered but the complex is less readily excreted by the kidneys producing a positive calcium balance.

TABLE 9

Blood phosphatase in bone diseases

(After Kay (15) 1932)

Conditions	Number of cases	Average phosphatase content of the plasma
		units
Normals	33	0.14
Infantile rickets	13	1.03
Adolescent rickets	1	2.4 or more
Renal rickets	2	1.2
Fragilitas osseum (infantile) and children	6	0.41
Osteitis fibrosa (generalized)	3	1.8
Osteitis fibrosa (local)	7	0.21
Osteitis deformans	24	1.7
Osteomyelitis	8	0.27
Arthritis with bony changes	7	0.17

Phosphatase

In 1932 Robison (14) reported the discovery of an enzyme, phosphatase, in the blood and able to split inorganic phosphate off of such phosphoric esters as hexose phosphates and glycerophosphates. Kay (15) studied the concentration of this enzyme in the plasma of both normal individuals and others with various bone diseases (see Table 9). According to Kay, this phosphatase enzyme performs no useful function in the blood and its presence there indicates leakage from the tissues. According to his theory, it increases the supply of inorganic phosphate by breaking down the phosphoric esters at the site of bone formation. Vitamin D can reverse this leakage and restore phosphatase to the bone site with resulting production of an effective solubility product (see Table 10).

Increase of serum phosphatase over the normal is said to be the earliest diagnostic sign of rickets. The increase may reach 20 times the normal content. However, it is generally agreed that return of serum phosphatase to normal as a result of vitamin D therapy does not insure that the rickets is completely cured. It indicates restoration of bone forming cells to normal activity but not complete restoration of bones to normality.

Vitamin D Functions

In 1940 Dr. E. A. Park stated: "We know nothing of how vitamin D acts." If we interpret that as exact manner of accomplishing effects the statement is still true today. There are however certain effects directly traceable to vitamin D action, for example:

TABLE 10

Influence of vitamin D from several sources on the serum phosphatase of chicks

(After Correll and Wise (16) 1938)

Time	Amount of vitamin D per 100 grams of diet and units of serum phosphatase			
	None	18 I.U. as cod liver oil	37 I.U. as cod liver oil	37 I.U. as tuna liver oil
On first day	71.3	71.3	71.3	71.3
In 2 weeks	158.7	56.4	69.6	81.3
In 4 weeks	267.7	44.1	41.4	65.0
In 6 weeks	248.0	54.8	48.2	115.2
In 8 weeks	240.0	44.0	38.6	76.6

N.B. Note the consistent reduction in serum phosphatase with cod liver oil in contrast to the effect of tuna liver oil. Explained by the superiority of the vitamin D_3 in cod liver oil over the D_2 in tuna liver oil for the chick. (For discussion of differences see page 47.)

1. In the gut vitamin D increases the absorption of calcium into the blood and to a lesser degree the absorption of phosphorus. For calcium absorption vitamins D_2 and D_3 are effective in amounts as low as one unit per gram of diet. Using radio active phosphorus (P32) Kaplan and Greenberg (17) showed that the absorption of phosphate given by stomach tube was 10–15 per cent but at the bone site the labelled phosphorus increased by 40 per cent. This increase showed that the effect of vitamin D on phosphate utilization was not entirely accounted for by the effect on calcium absorption.

2. Vitamin D in some way affects the retention of calcium and phosphorus. The Food and Nutrition Board of the National Research Council based its allowances of calcium primarily on the amount necessary to secure

normal retention of calcium and phosphorus in infants and children (see Table 11).

3. Vitamin D has a definite effect on the blood and bone concentration of phosphatase.

4. To be effective vitamin D requires at least a certain minimal quantity of both calcium and phosphorus in the diet.

The Council of Pharmacy and Chemistry of the American Medical Association recognizes that vitamin D therapy is a specific treatment for infantile rickets, infantile tetany and osteomalacia; for these it is both preventive and curative. There is evidence that vitamin D is important in tooth formation and also helps in the prevention of tooth decay. Data regarding the action of vitamin D on tooth decay (caries) have been given by Mellanby (18) and by MacBeath et al. (19) (see Table 12).

TABLE 11

Suggested daily allowances of calcium and vitamin D

(Food and Nutrition Board, National Research Council, 1948)

Individuals	Calcium	Vitamin D
	grams	*units*
Men (adults)	1.0	*
Women (adults)	1.0	*
Women (last ½ pregnancy)	1.5	400–800
Women in lactation	2.0	400–800
Girls 13–15 yrs.	1.3	400
Girls 16–20 yrs.	1.0	400
Boys 13–15 yrs.	1.4	400
Boys 16–20 yrs.	1.4	400
Children of either sex under 12 yrs.		
Under 1 yr.	1.0	400
1–3 yrs.	1.0	400
4–6 yrs.	1.0	400
7–9 yrs.	1.0	400
10–12 yrs.	1.2	400

* For adults who have no opportunity for exposure to clear sunshine and for elderly persons, the ingestion of small amounts of vitamin D may be desirable. Other adults probably have little need of vitamin D supplement to the diet.

Presnall (20) showed that the skin of rachitic rats had a lower oxygen consumption than that of normal rats and claimed that addition of vitamin D by mouth or topically increases skin respiration. Dalldorf (21) showed that addition of vitamin D to a culture medium of iris epithelial cells in vitro would increase their proliferation provided the medium was adequate in vitamin A; indicating a role of vitamin D in cell metabolism.

Acne and Vitamin D

Dotorsky and Platt (21) reported that vitamin D therapy reduced pustule formation in acne, and Henrichsen and Ivy (22), basing their opinion on the treatment of 210 cases with two levels of dosage (20000 and 30000 units daily) reached the conclusion that, while it is not a specific remedy for acne, it is of value in the treatment of that condition.

TABLE 12

Effect of vitamin D on tooth decay

I. Mellanby experiments

Dosage	Per cent of caries			
	1st inspection	Final inspection	Increase	Per cent increase
A. Sheffield tests				
On olive oil (no D)	25.96	35.96	10.00	62.13
On cod liver oil	42.86	44.41	1.55	10.28
On vitamin D concentrate	45.13	46.14	1.01	6.57
B. Birmingham tests				
On olive oil (no D)	15.59	23.22	7.63	45.85
On cod liver oil	19.26	22.23	2.97	9.81
On vitamin D concentrate	22.18	24.27	2.09	10.12

II. MacBeath experiments: Three groups of boys studied aged 10–14 years

Group	Number of boys	Dosage	Per cent increase in caries	Per cent increase of controls over treated gr.
I (controls)	23	No added vitamin D	2.83	
II	21	15 drops viosterol	1.73	1.63
III	19	Ultra-violet irradiation	0.32	8.84

N.B. The basal diet was the same for all the groups, viz.:

Protein	15.49% of the	Total calories: 1704
Calcium	0.628 gms.	Vitamin A: 3589.6 units
Phosphorus	1.132 gms.	Vitamin D: 19.1 units
Ca/P ratio	0.59	

Other skin affections

Cornbleet and Struck (23) report that vitamin D acts favorably on scleroderma; Ceder and Zon (24) on psoriasis; McLaughlin (25) as a treatment by topical application for X-ray burns; Comel (26) for treatment of eczema.

Relation of Vitamin D to Parathormone

The calcium content of the blood is controlled in part by the solubilit and absorbability of calcium salts supplied by the diet and in part by th parathyroid gland secretion (parathormone). Maintenance of normal bloo content of calcium is important; reduction below a certain level produce tetany. Removal of the parathyroid glands reduces serum calcium to abou one-half normal and tetany results. The action of the parathormone i governed in part by calcium and phosphorus metabolism. Administratio of calcium salts by mouth or intravenously abolishes symptoms of tetan raises the serum calcium and increases phosphate excretion, mainly i the feces.

But vitamin D can also be used to help combat a tendency to tetan It was therefore natural to suspect that it might act upon or through th parathyroids.

Taylor et al. (27) have postulated such an action. They base their claim on the observation that when parathyroidectomy in dogs is supplemente by thorough dissection of the neck to remove accessory parathyroid tissue the dogs so treated are not protected against tetany by vitamin D administration. Their statement has been both supported and challenged.

Reed, Struck and Steck (28) have reviewed the evidence pro and co and state:

> "There is strong evidence that vitamin D acts by stimulation of the parathyroi glands although the conclusions drawn from much of the evidence cannot be supported. There is also equally convincing evidence that the action of vitamin D and o parathormone differ in significant ways. Much of the argument pro and con disregard the fact that there are factors in no way related to either vitamin D or parathyroi which are capable of profoundly influencing calcium-phosphorus metabolism. A decision cannot be made until all of these factors are properly correlated."

Relation of Vitamin D to the Thyroid Glands

Reed et al (28) claim that vitamin D is intimately related to thyroi function in that deficiency increases the metabolic rate and is capable o elevating the metabolic rate in normal persons [Presnall (20)]. They suggest that this effect is not mediated through the parathyroids but probabl through the thyrotropic mechanism of the anterior pituitary.

Bennholdt-Thompson (29) has reported observations on the interrelatio of iodine, thyroid and vitamin D (see Table 13). It is generally recognize that goiter results from a deficiency of iodine. It has also been demonstrated that while elevated serum calcium does not increase the thyroi weight, a combination of calcium chloride and vitamin D has a significan goitrogenic effect. Calcium chloride without vitamin D does not have thi effect. Bennholdt-Thompson observed that vitamin D and ultra-viole irradiation both decreased the iodine content of the thyroid and increased the iodine content of the blood, irradiation being more effective than a vitamin D concentrate.

Vitamin D and the Eyes

Keratoconus, an eye disease, is characterized by a distention of the cornea of the eye. Blackberg and Knapp (30) report vitamin D therapy beneficial as a treatment.

Vitamin D and Arthritis

Vitamin D in large doses has been reported beneficial in certain types of arthritis. Theoretically one might hope that it would help in reducing the deposits of calcium in the hypertrophic type. Reports of tests have been conflicting, and there is some danger of bad effects of the large dosage usually employed in such treatment. (For the discussion of massive dosage see Section III.)

TABLE 13

Effect of vitamin D and ultra-violet light irradiation on blood iodine concentration

(After Bennholdt-Thompson and Wellman (29) 1934)

Test rats	Per cent iodine in	
	100 ml. blood	100 mg. thyroid
Controls	25.2	211
Irradiated 15 minutes daily	40.5	112
Fed 0.05 cc. Vigantol daily	31.0	167

Correlation of Vitamins A and D

Copp and Greenberg (31) have reported a study of healing in standard fractures of the rat fibula made by measuring the uptake of activated strontium by the callus and by measurement of the breaking strength of fractured bone. They found that in normal rats the most active calcification of the callus extends over a period of 8–16 days and the broken bone attains normal strength in 12–16 days.

In vitamin A deficient rats the callus is smaller than in normal rats and calcification less active. There was a distinct delay in fracture healing. This may have been an indirect effect of retarded growth activity when vitamin A was deficient. In rachitic rats there was no significant calcification of the callus unless vitamin D was added to the diet.

In the Steenbock-Black rachitogenic diet shown in Table 8 (used today to produce rickets in rats used in the U. S. Pharmacopoeia test for vitamin

D) there is provision for adequate vitamin A content to insure growth, and one of the conditions of the test is that the test animals must show growth during the test period. In fact it is possible to delay appearance of rickets by starvation.

Mellanby (1) has suggested that vitamins A and D work together in production of bone growth, that vitamin A controls the activity of the osteoblasts that lay down the soft bone, and that vitamin D governs the deposition of calcium phosphate to harden it.

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SECTION II. FORMS AND CHEMISTRY OF VITAMINS D

Vitamin D (all forms) is, like vitamin A, a fat-soluble vitamin. Modern investigation of its relation to rickets began in the years 1914–1918. Mellanby (1) studied the effect of various diets on puppies. He could produce rickets by feeding them milk and bread or oatmeal porridge. At that time an emulsion of linseed oil was widely sold in England as a cure for rickets and, though found useless for this purpose, it stimulated the study of other fats and oils. Cod liver oil proved at the time the best antirachitic fat investigated.

As stated in Section I, Hess and Unger (2) showed that the antirachitic factor in cod liver oil could not be vitamin A, and in 1921 McCollum et al (3) demonstrated the presence in cod liver oil of a true antirachitic factor to which they assigned the name vitamin D.

Bills (4) has reviewed the progress in isolation and identification of the vitamin. He lists the following as the five crucial findings leading to chemical identification of the vitamin:

1. McCollum's (3) proof that cod liver oil deprived of its A content by oxidation retained its antirachitic potency.
2. Hess' (5) and Steenbock's (6) simultaneous discovery that irradiation of certain foodstuffs by ultra-violet light made them antirachitic in effect.
3. Hess' (5), Steenbock's (6) and Rosenheim and Webster's (7) demonstrations that the sterol fraction of oils and foodstuffs became antirachitic on irradiation.
4. Windaus and Hess' (8) identification of the ergosterine of Tanret (9) as the parent substance of a vitamin D.
5. Isolation of calciferol (irradiated ergosterol) by Angus et al. (10) and Windaus et al. (11).

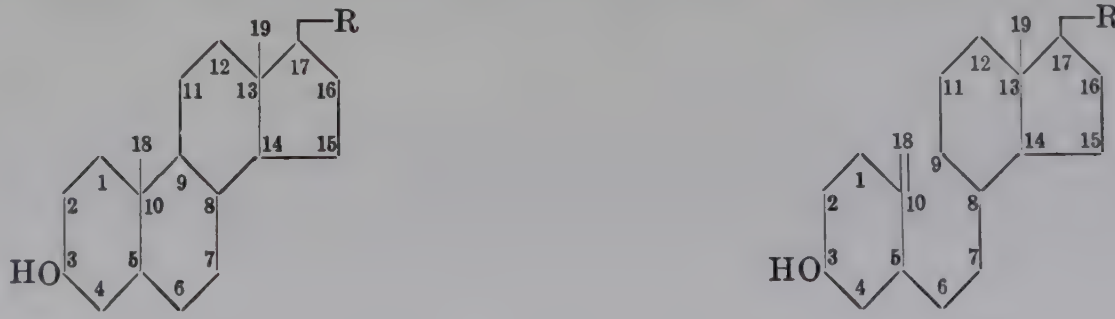
For forms of vitamin D see figure 4.

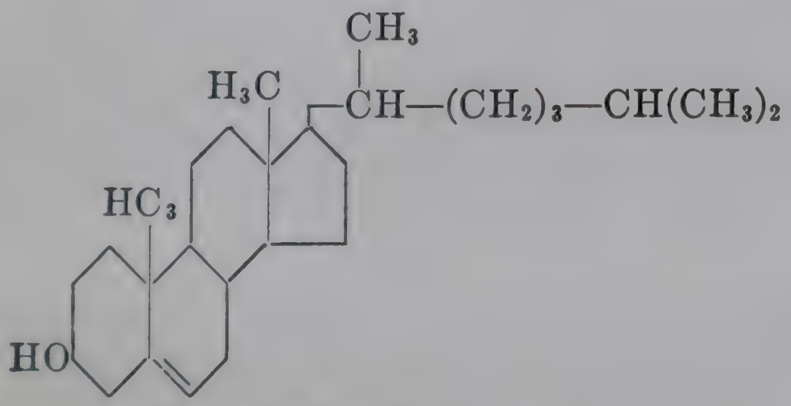
Ergosterol and Calciferol

In 1879 Tanret (9) isolated from the fungus ergot a compound to which he gave the name of ergosterine. This was the first provitamin D to be identified by Askew et al. (10) and Windaus (11). Its conversion to vitamin D_2 or calciferol was accomplished by irradiation with ultra-violet light waves, but such conversion is possible by action of cathode rays [Knudson and Moore (12)] and by radium emanations [Moore and Devries (13)].

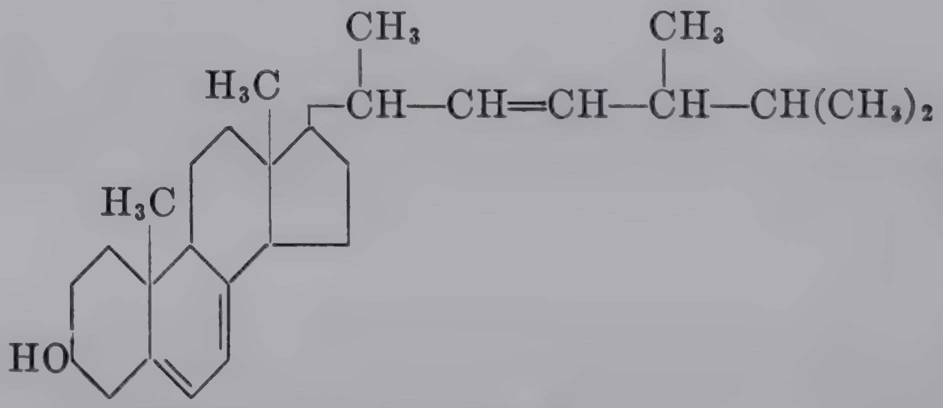
But it was soon discovered that irradiation of ergosterol converts only about 50 per cent into calciferol. In the process a series of compounds are formed which in the order of their appearance are: calciferol or vitamin D_2, lumisterol, tachysterol, Toxisterol, and two supra-sterols I and II. Of these, calciferol, lumisterol, and the two supra-sterols have been isolated in crystalline state. Tachysterol is also known today as A.T. 10, the initials

Positions in the sterol nucleus and breaking of 9–10 bond in process of activation

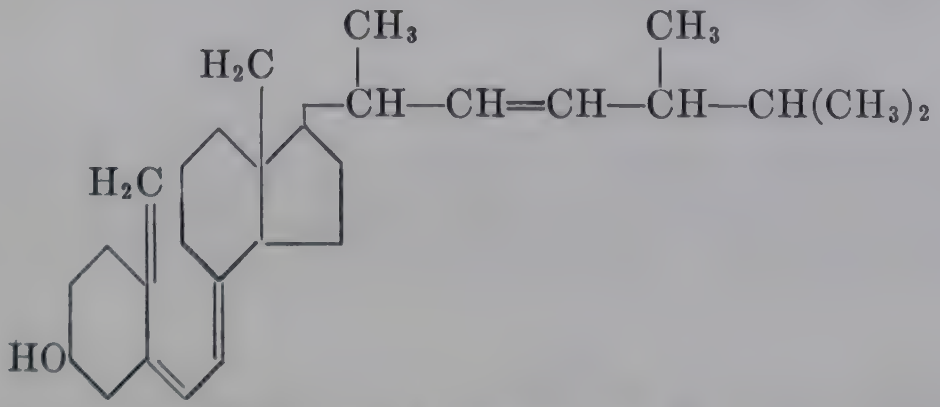




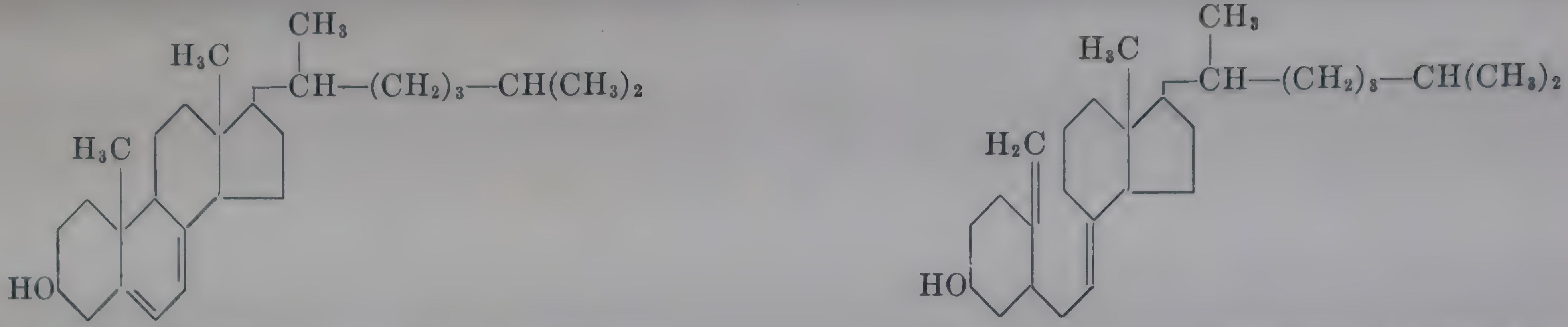
Inactive cholesterol



Ergosterol; Provitamin D_2



Calciferol; Active vitamin D_2



7-Dehydrocholesterol; Provitamin D_3 (inactive)

Activated 7-dehydrocholesterol; Vitamin D_3

FIG. 4. FORMS OF VITAMIN D AND THE STEROL NUCLEUS

A.T. standing for "anti-tetany" as it is used in the treatment of tetany. Of all these compounds formed by irradiation of ergosterol, only calciferol (Vitamin D_2) has appreciable antirachitic potency. Lumisterol can be converted into calciferol and may form with it an addition compound consisting of one part lumisterol and one part calciferol. It was this addition compound that was called vitamin D_1 by German investigators. After this was discovered the term Vitamin D_1 was dropped and calciferol became vitamin D_2. For data on the steps in these discoveries see Linsert (14), Windaus and Luttringhaus and Deppe (15), Windaus, Linsert, Luttringhaus and Weidlich (11).

Toxisterol gets it name from the report that it was toxic by Laquer and Linsert (16). One of earliest patented vitamin D preparations made in Germany and called vigantol occasionally produced toxic effects. These were explained by failure to remove toxisterol from the irradiation products.

Just what happens when ergosterol is converted to calciferol to give it its antirachitic effect is still not fully understood. It is known that irradiation opens the sterol ring between positions 9 and 10 (see figure 4) but activity is also affected by the character of the side chain, for example the difference between vitamins D_2 and D_3.

Pure, anhydrous ergosterol has the chemical formula $C_{28}H_{43}OH$; a melting point at 166°C.; absorption bands at 260, 270, 282, 293.5 $\mu\mu$; $(\alpha)^{20}_{5461} = -171°$; $(\alpha)^{20}_{D} = -132°$ in chloroform. Calciferol (irradiated ergosterol) has an identical chemical formula, $C_{28}H_{43}OH$; melts at 115–118°C., has a strong ultra-violet absorption with a maximum at $264\mu\mu$; $(\alpha)^{20}_{5461} = +122°$ and $(\alpha)^{20}_{D} = +102.5°$. Its antirachitic potency is 40,000 U.S.P. units per mg.; i.e. .000025 mg equals 1 U.S.P. or International unit.

Viosterol is a solution of calciferol in oil containing not less than 10,000 U.S.P. units per gram of Vitamin D_2.

Vitamin D_3; 7-dehydrocholesterol

The antirachitic substance in cod liver oil is present in the non-saponifiable fraction which consists largely of cholesterol. In 1935 three laboratories (17) confirmed the hypothesis that provitamin D was an "impurity" in ordinary cholesterol. Bills, Honeywell and McNair (18) also confirmed this finding and the belief arose that the "impurity" was ergosterol. This assumption was later proven erroneous. The studies that led to the new viewpoint came from contrasting the effect of irradiated ergosterol and cod liver oil on rats and chicks. As early as 1930 Massengale and Nussmeier (19) found it necessary to administer the equivalent of 200 per cent cod liver oil in the form of irradiated ergosterol to produce in chicks the effect of 2 per cent cod liver oil itself. Similar results were reported by other investigators (20 and 21). In 1934 Waddell (22) tested irradiated cholesterol as a rickets preventive for chicks and proved it more effective than an equivalent number of rat units of irradiated ergosterol. The difference was due to the fact that the cholesterol "impurity" in cod liver oil was not ergosterol but another similar sterol, namely 7-dehydro cholesterol or provitamin D_3. Isolated by Windaus et al. (23), it now appears to be the principal vitamin D not only in cod liver oil but also in the human skin.

Provitamin D_3 is identical with ergosterol chemically except that it lacks the methyl group in the side chain and one double bond. In that respect it differs from the vegetable sterols. Its melting point is 84–85°C.; $(\alpha)_D^{20} = +51.9$ in 1.6 per cent solution of chloroform, $+84.8$ in acetone. It crystallizes in white needles. The absorption spectrum is similar to that of ergosterol.

Vitamins D_2 and D_3 appear about equally as potent as antirachitics for rats and for infants. For chicks D_3 is definitely more effective than D_2 (McChesney (24)). McChesney found that when given orally in oil, the requirement for chicks was 24 rat units of D_3 and 850 units of D_2, or approximately one part of D_3 equivalent to 35 parts of D_2. Correll and Wise (25) put the ratio of D_2/D_3 for chicks as 30/1. McChesney has suggested that the superiority of D_3 over D_2 for chicks may lie in the fact that the chick absorbs D_3 better than D_2. D_3 is also slightly superior to D_2 for rats, 1 part of D_3 being equivalent to 1.31 parts of D_2.

Other forms of vitamin D

Vitamins D_2 and D_3 are the two forms of special significance in human and animal nutrition. There are, however, other antirachitic substances but they are of interest mainly to the chemist. Their antirachitic potency is low compared to that of D_2 and D_3. Vitamin D_4, or activated 22-dihydro calciferol, may be the form that is activated when cereals are irradiated. Vitamin D_5 or 7-dehydro sitosterol, is slightly antirachitic for rats. Vitamin D_6 is the name applied to cholesterilene sulfonate, first prepared by Bills (4) by treating cholesterol with fullers earth. It is slightly more effective for chicks than for rats and does not require activation by ultra-violet light.

The literature contains descriptions of other antirachitics, e.g. 7-hydroxy cholesterol [Bills (4)]; 22, 23 oxide ergosterol (Windaus and Trautman (26)); a product prepared by treatment of ergosterol with nitrites; an irradiated product prepared by heat treatment of 7-keto cholesteryl acetate and isobutyl magnesium bromide (Windaus and Kharasch (27)); and irradiated cholesterol after freeing from normal provitamin D_3 but not heated (Stavely and Bergman (28)). For details consult Bills' review (4).

A.T. 10

In 1934 Holtz et al. (29) described a product with little antirachitic potency but superior as an antitetanic agent to either parathyroid extract or vitamin D in that it could be administered orally in smaller dosage than vitamin D to attain the desired effect. The commercial product is called A.T. 10, the initials A.T. standing for "anti-tetany". Chemically it appears to be dihydro-tachysterol, one of the products produced by irradiating ergosterol. For comparison of the effects of A.T. 10 and vitamin D see Tables 14 and 15.

TABLE 14

Effect of vitamins D_2, D_3, and A.T. 10 on bone ash of the chick

(After McChesney (24) 1943)

Vitamins	Route of administration	Vehicle	U.S.P. units required per week per chick
D_2	Oral	Corn oil	850
D_3	Oral	Corn oil	24
A.T. 10	Oral	Corn oil	6
D_2	Intramuscular	Corn oil	8000
D_3	Intramuscular	Corn oil	90
A.T. 10	Intramuscular	Corn oil	75
D_2	Intravenous	Propylene glycol	110
D_3	Intravenous	Propylene glycol	9
A.T. 10	Intravenous	Propylene glycol	10

TABLE 15

Control of blood calcium and phosphate

(After Albright et al. in Shohl's Mineral Metabolism, Reinhold Pub Co., N. Y. City, 1939)

Agents	Ca in serum	PO_4 in serum	Ca absorption	PO_4 excretion
Vitamin D	+	+++	+++	+
A.T. 10	++	++	+	++
Parathormone	+++	+	0	+++

Ertron

Another commercial form of vitamin D is called "ertron". It is ergosterol activated by the Whittier (31) process. The etherial solution of the activated material is dried on casein to provide capsules claiming 5000 U.S.P. units per capsule. It has been used in the attempt to alleviate arthritis of certain types.

Stability of Vitamin D

Huber and Barlow (32) report a study of the stability of vitamin D_2 and D_3. They found that under storage conditions D_3 is somewhat more stable than D_2; that stability of crystalline D_2 and D_3 in the presence of air, nitrogen or CO_2 is less than when stored in an evacuated capsule. In such capsules D_2 underwent no change in 9 months, D_3 in 12 months. Solutions in propylene glycol or vegetable oil retain their potency over long periods of time at a temperature of 4 degrees.

Fritz et al. (33) have shown, however, that vitamin D can undergo oxidative destruction and that rise in temperature tends to increase the rate of such destruction. The type of carrier is also important; cereal carriers tend to protect, while mineral mixtures and sugar accelerate destruction.

TABLE 16

Effectiveness of various wave lengths of light for healing rickets and activating provitamin D

(After Knudson and Beriford (34) 1938)

Wavelengths	Energy to produce 2 + healing	Energy to form one unit of vitamin D	Efficiency compared with wavelength 2804
Angstrom units	*ergs*	*ergs*	*per cent*
2653	948,000	287,000	79
2804	774,000	226,000	100
2894	1,305,000	395,000	57
2967	927,000	280,000	81
3024	1,976,000	353,000	39
3128	91,000,000	27,545,000	1

TABLE 17

Methods of expressing vitamin D potency

1 U.S.P. unit equals 1 International unit equals 0.000025 milligram calciferol

1 International unit equals 3.25 A.D.M. A units

1 International units equals 0.37 Steenbock units

A product labelled 250D equals 250 times the amount in the cod liver oil used as the reference standard; usually a cod liver oil containing 85 U.S.P. units of vitamin D per gram

Rancidity in the carrier oil also enhances destructive activity, but exposure to light is without effect.

Irradiation Values

Active vitamins D are not only producible by irradiation but the effect varies with the wave length. Knudson and Beriford (34) have calculated the energy in ergs of different wave lengths necessary to produce rickets healing effect and activation of provitamin D (see Table 16).

Methods of Expressing Vitamin D Potency

Potency of vitamin D is determined by bio-assay (U. S. Pharmacopoeia XIII, 1947). As a reference standard a cod liver oil containing 850 units of vitamin A and 85 units of vitamin D has been used and there are various ways of expressing potency as shown in Table 17. A physico-chemical method would be highly desirable and such procedures have been reported [Ewing et al. (35) and DeWitt and Sullivan (36)]. At present, however, reliance is placed on the bio-assay method for checking claims for potency.

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SECTION III. EVALUATION OF VITAMIN D FOR HUMAN AND ANIMAL USE.

In discussing the bases for the daily allowances of vitamin D suggested by the Food and Nutrition Board of the National Research Council (See table 18) Jeans (1) says:

> "In judging the appropriate amount of vitamin D for infants we find some conflict between the interpretations of clinical experience in large scale out-patient studies and interpretations of smaller but better controlled groups. The latter type of study gives results indicating that 300 to 400 units daily is a fully adequate amount for infants. At this level of intake linear growth of infants exceeds the average in rats; retentions of calcium and phosphorus are excellent; dentition occurs earlier than usual. Any amounts greater than 300 to 400 daily do not increase the calcium and phosphorus retentions above those observed with the smaller amount. Yet some confusion has arisen because of criteria used in the interpretation of the roentgenograms by a few observers. On the basis of the structure of the metaphysis of the long bones, the diagnosis of rickets has been made in the case of infants receiving 300 and 400 units of vitamin D. Naturally, if rickets occurs with this amount of vitamin D a larger amount is indicated. However, these bone changes do not occur with 135 units of vitamin D in milk, a much smaller amount and one that produces smaller retention of calcium and phosphorus. A more appropriate interpretation is that the bone changes observed with 400 units are not rachitic and probably are concomitants of the rapid growth that occurs with this intake. It was because of this conflict of opinion that the range 400–800 units was stated in the table of allowances.
>
> The vitamin D requirement beyond infancy is known with less certainty than for infants, but the values in the table of allowances are believed to be ample. Children beyond infancy who are receiving 300 to 400 units of vitamin D daily have retentions of calcium that correspond to normal as determined by calculations based on rates of growth."

Another reason for exceeding the 400 unit allowance has been advanced by MacBeath and Verlin (2) who claim that for the prevention of tooth decay at least 800 units of vitamin D daily are necessary and that D_3 is more potent than D_2.

In Section II the relative value of vitamins D_2 and D_3 for chicks and rats was discussed (see page 47). Their relative value for humans has also been studied. Harnapp (3) reported that 5 mg. of D_3 in massive dose had the same prophylactic effect as 7.5 mg. of D_2; a ratio of D_3/D_2 of 1/1.5. However relative values of D_2 and D_3 for human needs require further study.

Jeans and Stearns (4) state that it must be given in an amount to produce normal growth and mineralization of the skeleton including the teeth and bones of infants and children; for maintenance of their structures in sound condition throughout adult life; and to supply the special needs of pregnancy and lactation (see Table 11).

FOOD TECHNOLOGICAL RESEARCH

As noted in the footnote to Table 11, the question of requirement for th average adult, man or woman, is unsettled, but certainly does not excee 400 units per day. Though rickets is primarily a vitamin D deficiency disease of infancy, the table gives allowances for boys and girls up to 20 year of age. In that connection Follis et al. (5) suggest vitamin D supplementation of diet be continued at least to the 14th year, e.g.:

> "We doubt if slight degrees of rickets, such as we found in many of our children interfere with health and development, but our studies as a whole afford a reason t prolong the administration of vitamin D to the age limit of our study, the 14th year and especially indicate the necessity to suspect and take measures to guard agains rickets in sick children."

It must also be emphasized that the vitamin D requirement is conditione by the calcium and phosphorus content of the diet and that vitamin D i not a substitute for calcium and phosphorus adequacy in the diet. Th behavior of these elements as the minerals in the diet is controlled b vitamin D, the vitamin is not a mineral substitute.

Signs of Vitamin D deficiency

The Council on Foods and Nutrition of the American Medical Association lists the following stigma as evidence of vitamin D deficiency: "Bowe legs, malformation of the chest (funnel breast), and defects of the teet may be residues of early rickets which is no longer active or susceptible t treatment, enlargement of the wrists, elbows, knees, ankles, and costachondral junctions (beading and rachitic rosary), bulging forehead (crania borses)."

The Council suggests for treatment of rachitic infants 1500–2000 unit of vitamin D daily continued for several months and double this amoun for premature infants.

Selection of Forms of Vitamin D

As already stated there is some evidence that for infants vitamin D (the form predominant in cod liver oil) is somewhat more efficient tha vitamin D_2. However, in evaluating available offerings of vitamin D i irradiated, fortified or metabolized milks, in irradiated yeast, in viosterol or in other fish liver oils or other irradiated and fortified foods it is safe t build allowances on unit content, regardless of whether the vitamin presen is D_2 or D_3.

Unlike most of the other vitamins, vitamin D is contained in only a fe natural foods; namely in certain oily fishes and in eggs. Herring, sardines tuna, and salmon are fairly rich in the vitamin. Eggs of all kinds contai some. Fish roe are the richest of these egg forms. The amount in hens egg is not great and very variable. It is said to take at least 5 egg yolks to furnish the vitamin D equivalent of one teaspoon of cod liver oil.

With the exception of vitamin D milks, then, there is little reliance to be placed on ordinary articles of diet as a source of vitamin D. This means that supplements must be supplied to infants and children. One teaspoon of cod liver oil supplies approximately 350 units of vitamin D. One mg. of crystalline calciferol gives 40000 units and in the form of viosterol, 10000 units per gram.

"Vitamin D Milks" vary in content according to the method of production. They are of three types; irradiated, metabolized and fortified milks. Irradiated milk is raw milk irradiated with ultraviolet light; the potency averages about 135 units per quart. Metabolized milks are produced by feeding the cow specified amounts of irradiated yeast. The amount of D the cow extracts and transfers to her milk varies with the daily intake of the yeast but, in general, such milks are built to provide about 400 units per quart. Certified milk producers have used this method of fortification extensively. Fortified milks are milks to which a vitamin D concentrate is added. Again the amount depends on the amount of concentrate added, but 400 units per quart is the usual addendum. Raw milk is not rich in vitamin D, but human milk is superior in this respect. However, human milk does not supply enough to breast fed children and cod liver oil supplementation is necessary and should be begun at an early age.

According to Park (6):

> "Since the period of greatest susceptibility of rickets is the first months of life, it is important that full dosage of vitamin D be reached early, certainly by the end of the second month. If cod liver oil is selected it should be started at the beginning of the third week or at the latest the fourth week with a dose of one half teaspoon (175 units). This dose can be increased to one teaspoon (350 units) after a few days. In the course of the next two weeks it should be raised to 2 teaspoons (700 units). This dosage may be enlarged to 3 teaspoons or allowed to stand according to the particular case. It is certainly advisable to give 3 teaspoons if for any reason the infant seem particularly liable to rickets. The dosage of 700–1000 units should be continued throughout the first year. As much as 700 units should be continued throughout the second year."

For the cure of rickets larger doses are necessary. If cod liver oil is used at least 3 teaspoons daily and, if larger amounts are necessary, it is best to use the concentrated forms of vitamin D under the direction of the physician.

The "Stoss" Therapy

The Stoss treatment was suggested by Harnapp (7). The treatment involves (instead of daily dosage) the giving of a single massive dose of vitamin D (200,000–400,000 units). Harnapp claims that such a massive dose given to infants and children at the beginning of the winter months will provide protection over the entire winter month period. This plan, howeve has not met with general medical acceptance in this country mainly fro fear of untoward effects following too high a dosage.

Vitamin D Toxicity

In reply to a query as to vitamin D toxicity the Question and Answe section of the American Medical Journal gave this reply:

> "An excessive dose is a relative matter that can be determined only by trial. Som individuals are reported to have tolerated well for considerable periods daily doses a high as 30,000 units per kilo of body weight. However, these responses appear not t be true hypervitaminosis D but rather gastrointestinal sensitivity to the solvent.

Park (6) states:

> "The rule holds that the dose will not become toxic so long as the calcium and inorganic phosphate levels of the blood are not affected. Apparently the toxic actio does not depend upon the level of vitamin D in the blood but rather on its effect o the calcium and phosphorus metabolism."

Park also suggests that one should be on the lookout for untoward effect if the daily dose exceeds 20,000 to 30,000 units daily for infants; 50,00 units for children or 200,000 units for adults.

Since the daily requirements (preventive) do not exceed 800 units dail there is little danger of excess vitamin D when taken as a supplement t diet within the prescribed allowances (see table 11). Trouble can develo when using the "Stoss" plan or with the massive doses sometimes used i treatment of arthritis. Reed et al. (8) suggest that the daily oral administration of 10,000 units per pound of body weight is the upper limit and the danger is less if such daily doses are divided.

The following toxicity comparisons have been made: toxicity with cod liver oil at 50,000 times the requirement; with D_2 at 1000 times the requirement and with A.T. 10 at 10 times the requirement. In actual amounts the border line toxic doses appear to be at 50 mcg. for D_2, 60 mcg. for D_3 and 6 mcg. for A.T. 10.

Effect of Cereals in the diet on Vitamin D Requirement

As early as 1921 Mellanby (9) called attention to the fact that dogs an rats were prone to develop rickets and poor teeth when fed diets high i cereals. This effect was at first ascribed to the greater growth produce when cereals were added to the diet. The extra growth was supposed t create a demand for more calcium and vitamin D to assure normal bon formation. However, in spite of the fact that they contained more calciu and phosphorus, oatmeal and wheat germ interfered more with bone calcification than rice or white flour. This led Mellanby to the idea of an anticalcifying factor in certain cereals, the "toxamin theory."

In 1927 Holst (10) reported that the anticalcifying factor could be removed by extracting the cereals with cold, dilute hydrochloric acid. Bruce and Callow (11) were the first to suggest that phytic acid (inositol-hexaphosphoric acid) or its calcium magnesium salt might be the ingredient in cereals responsible for the rachitogenic effect.

Further information came from Harrison and Mellanby (12). They reported: 1) Phytin (Ca-Mg-Phytate) does not have rachitogenic properties; 2) Phytic acid or sodium phytate is as strongly rachitogenic as oatmeal fed in amount to provide equivalent total phytate; 3) Sodium phytate prepared from oatmeal retains the full rachitogenic activity of the oatmeal and further purification does not decrease the activity of the phytate; 4) If sodium phytate is hydrolysed by boiling at 100°C. with hydrochloric acid the rachitogenic effect disappears and the same is true of oatmeal treated likewise; 5) The rachitogenic effect of sodium phytate as well as that of oatmeal is counteracted by calcium.

Harrison and Mellanby analyzed cereals for phytin and phytate and found that of the total more than 50 per cent is phytate. Since the rachitogenic effect of cereals is counteracted by increasing the calcium in the diet it should follow that the anticalcifying effect of a cereal should depend on its calcium content as well as its phytate content; the phytate/calcium ratio should be the important factor. But a contradiction arose. Oatmeal contains about the same content of phytate as wheat and more calcium and phosphorus and yet wheat does not produce rickets and oatmeal does.

McCance and Widdowson (13) found the answer in the behavior of the enzyme phytase. They showed that oats contain little of this enzyme compared with wheat. Due to this enzyme wheat is able to hydrolyse 50 per cent of added phytate within 12 minutes while oats require eleven to thirteen hours. Again, before oats reach the consumer they are cooked directly, thus destroying what enzyme may be present. On the other hand, wheat and rye, used for bread baking do not get this destructive action; dry heat as high as 90 deg. does not inactivate phytate.

Mellanby (14) concurs in this explanation and notes that, although oats and wheat contain about the same amount of phytate, the relative phytate content of the two cereals before they reach the consumer is altered twice and the oat cereals actually used contain more phytate than the wheat products used. Difference in processing accounts for part of this difference. In preparing oatmeal the husk (containing little phytate) is removed and the dehusked meal rises in phytate concentration to about 270 mg. phytate per 100 grams of meal. On the other hand in the processing of wheat to flour the milling removes the bran which, unlike the oat husk, is rich in phytate. Result is that wheat meal flour has only about one third the phytate content of oat meal; 100–120 mg. per 100 grams.

Again, in preparing the cereals for eating oatmeal is boiled as porridg the small phytase content destroyed and the phytate content remain high. When wheat flour is used in bread making the large amounts of phytase present destroy the phytate during the proofing period and additio of yeast (rich in phytase) also accelerates the destruction.

The mystery of the rachitogenic effect of oatmeal was thus resolved Since the effect can be counteracted by calcium and extra vitamin D ther is an added reason for plentiful use of milk with the oatmeal porridge especially those vitamin D milks which supply both calcium and vitamin D

Incidentally, one of the recent develops in the dairy industry is the production of homogenized milk. In such milks the butter fat is broken dow into so small globules that it does not separate as cream on standing. Thi has special advantage when the milk is used for cereals or drinking for th reason that the fat-soluble vitamin D is in the butter fat. Homogenizin insures it uniform distribution throughout the milk.

Sunlight and vitamin D Activation

That the impinging of sunlight upon the bare skin can prevent ricket was first noted by Huldschinsky (15) in 1920. It was later shown that th explanation lay in the presence in the skin of provitamin D (mainly D_3) and that the ultra-violet rays of sunlight were the part of the light responsible for the activation. The sunlight which reaches the earth varies both in the length of the ultra-violet rays it supplies and also in their intensity. The limits are from 380 to 290 $\mu\mu$. According to Park (6) when th antirachitic power of individual ultra-violet wave lengths are tested by th monochromatic illuminator it appears that radiation at 313 $\mu\mu$ exerts only a slight effect, radiations at 302, 297, 280, 265, and 253 exerted a strong effect, those at 248 and 240 $\mu\mu$ a feeble effect and those at 237, 220 and 200 $\mu\mu$ no antirachitic effect whatever.

It is possible, then, for human beings and animals to secure their need of vitamin D by sun exposure as well as by taking vitamin D supplements. But it must be borne in mind that sunlight intensity varies with the season, being more potent in summer than in winter. Presence of dust or fog in the air acts to filter out the ultraviolet waves and diminish sun power. Ultra-violet waves do not pass through ordinary window glass or clothing and hence exposure to be effective must be of naked skin to direct sunlight or sky-shine.

These facts have resulted in the development of various ways to secure action of ultra-violet waves of suitable length and intensity without outdoor exposure. Lamps that can be used indoors have been developed to produce these waves; special types of window glass have been developed to transmit up to about 60 per cent of the sun waves. They have successfully brought the sun indoors, but remember that there are other advantages of getting babies into the open air beside antirachitic effect of sunlight. Daily exposure in such a way to make sunlight contact is still desirable; only bear in mind that the rays can also burn and damage skin and eyes if too intense, so turn the baby in such a way as to shade the eyes and clothe the unexposed parts of the skin to prevent colds.

Luce-Clausen (16) has reviewed the clinical aspects of ultraviolet therapy and gives these general conclusions as to its value: 1) Its use in prevention and cure of rickets and tetany is proven and both safe and specific if properly given; 2) It appears of little value in the treatment of bone fractures and its value in tuberculosis has been reported useful but no claims for specificity have been substantiated; 3) Because of its germicidal action it has proven of value in treatment of skin diseases provided the organisms lie within the range to which the rays penetrate and are killed or attenuated by doses safe for the host. In certain skin diseases (psoriasis?) a hyperemia produced by the rays may act for beneficial results.

Topical action of Vitamin D

The fact that vitamin A, topically applied, can be absorbed through the skin and function systemically was discussed in Chapter II (page 14). Hume, Lucas and Smith (17) in 1927 showed that the vitamin D in irradiated cholesterol could be absorbed from a small area of undamaged skin in sufficient amounts to supply the needs of the test animal; rickets was prevented in rabbits fed a rachitogenic diet and an almost normal calcification of the bones produced when an area of the skin 2.5 x 3.5 cm. was irradiated for ten minutes three times a week. These animal studies demonstrated both the effect of irradiation of small skin areas and absorption of vitamin D by inunction. By applying locally a salve fortified with viosterol, McLaughlin (18) treated some recalcitant X-ray burns successfully.

For general vitamin D therapy oral dosage or ultraviolet ray exposure of the skin is to be preferred to topical application, but in the case of certain skin lesions there appears to be merit in topical applications at the site of injury.

Vitamin D and Domestic Animals

According to the reports of the National Research Council's Committee on Animal Nutrition (19) vitamin D is an essential for all domestic animals, especially in the early stages of life. One report states:

> "In calves prolonged deficiency of vitamin D causes rickets. Clinical symptoms are usually preceded by a decrease in either or both blood calcium and phosphate. This is usually followed by poor appetite, decrease in growth rate or loss of weight, digestive disturbances, stiffness of gait, and occasional convulsions. Later, enlargement of

TABLE 18

Some suggested vitamin D allowances for domestic animals

(After Reports of the Committee on Animal Nutrition, National Research Council (19))

I. *Beef Cattle:*

Requirement for young calves 300 U.S.P. units per 100 lbs. of live wt. Unc usual conditions beef cattle receive sufficient vitamin D from exposure direct sunlight or by consumption of sun-cured roughages (Bechtel 20)).

II. *Dairy Cattle:*

Bechdel et al. (21) give the following allowances:

Weight of animal	Units of vitamin D daily	Grams daily of	
		Calcium	Phosphorus
lbs.			
50	150	4	2
100	300	8	6
150	450	12	10
200	600	13	12
400	1200	14	25

For larger dairy cattle vitamin D is required but no specific figures appe warranted as yet. When enough vitamin D is given for normal reproducti extra amounts do not stimulate milk production but do affect the vitamin content of the milk produced.

I

II. *Sheep:*

For sheep the allowances suggested for calves; 300 U.S.P. units daily per 1 lbs. of live weight probably adequate.

IV. *Swine:*

For swine the following allowances daily have been suggested:

Class of animal	Live weights	Ca	P	D
		grams	*grams*	*units*
Growing fattening pigs	50	7.4	4.9	135
	100	13.7	9.1	250
	150	15.8	10.5	330
	200	17.9	11.9	375
	250	17.9	11.9	415
Pregnant gilts & sows & young boars		16.4	10.9	300

V. *Poultry:* (Measured in A.O.A.C. units)

For starting chicks 180 units per pound of feed.

For laying and breeding hens 450 units per pound of feed.

For turkey poults 360 units per pound of feed.

For turkey breeders 450 units per pound of feed.

the joints, slight arching of the back, bowing of the legs, and erosion of the joint surfaces cause additional pain and difficulty in locomotion. Symptoms develop more slowly in other animals. Work with dairy cattle has shown that vitamin D deficiency in the pregnant female may result in dead, weak, or deformed calves at birth."

The effect of vitamin D deficiency on sheep and swine is similar.

Of poultry the report says:

"There is rickets and disturbed metabolism of calcium and phosphorus. Abnormal bone development is detected most readily in the legs and at the junction of the ribs on the sides of the breast. The spinal column may be curved and the sternum usually shows acute lateral bending or depression. There is enlargement of the hock joints and beading of the ribs. The beak is soft and bends easily. In mature laying hens the first symptom is the laying of thin shelled eggs followed slowly by actual decrease in egg production. The breast bone becomes soft and rubbery and the bones of the legs and wings become fragile and easily broken. Birds may lose temporarily the use of their legs and squat in a penguin-like manner, a symptom which has sometimes been called "egg paralysis". Hatchability of the eggs is markedly reduced. . . . The symptoms of vitamin D deficiency in turkeys are very similar to those in chickens."

In Table 18 are shown some of the suggested allowances for domestic animals, but for details consult the Animal Nutrition Committee reports (19.)

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CHAPTER IV. VITAMIN E

SECTION I. FUNCTIONS OF VITAMIN E

In his review of the status of Vitamin E in 1938–39 Mattill (1) wr as follows:

> "Some fifteen years ago several investigators in animal nutrition began to sus that for normal reproduction the rat required a dietary constituent not yet recogn or included within the generally accepted group of accessories. At first there was s hesitation in accepting yet another member into the already large family of vitan but convincing evidence from various laboratories, especially from that of Evans his coworkers, soon established the fact that when reared on diets otherwise comp but not containing this new fat-soluble factor, rats did not have offspring altho they appeared normal in other respects. Male animals became infertile through generation of the germinal epithelium and the damage was irreparable. Females fa to carry their young to term; the embryos died and were absorbed, but the fem reproductive mechanism as such was not damaged, since adequate dosages of missing factor restored fertility. Still other experiments showed that diets in wh this factor, although originally present, had been destroyed by oxidation were li wise inadequate for reproduction."

Mattill and Conklin (2) as early as 1920 had reported that female r fed on cow's milk were incapable of raising young even when the milk w supplemented with yeast and iron. Evans and Bishop (3) demonstra in 1922 the existence of a vitamin whose deficiency was responsible for t effect. At the time they called it "vitamin X" which was later changed vitamin E and still later, after chemical identification was achieved, tocopherol (Tokos, child birth; phero, to bear, and ol, alcohol). But in sp of extended study of the action of the vitamin on experimental anim Mason (4) comments: "Its chemical nature is known, its laboratory sy thesis accomplished, its wide distribution in the plant and animal wo recognized, and the effects of its absence extensively studied in a la series of laboratory animals. *Its physiological role in the animal body is s a matter of conjecture.*"

As would be expected from the studies that led to its discovery, attent was first concentrated on its effect upon male and female sterility.

Effect of vitamin E deficiency on the female rat

In the female rat vitamin E deficiency does not affect the processes estrus, ovulation, conception or implantation of fertilized ova. Th processes are normal in every respect. But, developing embryos and f membranes show abnormal development. These result in intra-uter death of the fetus followed by autolysis and resorption of the products conception. These fetal effects can be prevented by administering suffici vitamin E as late as ten days after conception.

According to Mason (4, 5) the cause of fetal death and resorption is due to abnormalities in the vascular system characterized by stasis, distention, and thrombosis (especially of the venous channels) which lead eventually to a local anemia with contraction of blood vessels (ischemia) and sometimes to hemorrhages which are the real causes of the fetal death. This effect is not prevented by administration of vitamins C or K but is reparable by vitamin E.

Effect of Vitamin E deficiency on the Male Rat

Permanent sterility results in male rats deprived of vitamin E. There is an irreparable degeneration of the seminiferous epithelium at the onset of sexual maturity but not during adolescence. Within several weeks practically no germ cells remain in the seminiferous tubules.

As Mattill (6) puts it:

> "The striking difference in response to vitamin E deficiency by male and female animals can be explained, superficially, by the fact that in the male the damage is done to a part of the animal's own tissue and may for this reason be irreversible in contrast to reparable damage in the female, which is wrought not on her own tissues but on those of the fetus."

Adamstone (7) expressed the opinion that vitamin E is intimately associated with the nucleus during cell division and probably exerts an indirect controlling influence.

The effect seen in the male rat does not occur in the mouse and there are species differences in response to vitamin E deficiency (see Table 19). Adamstone also reported that testicular degeneration resembling that in the rat occurs in chicks fed a natural diet with the vitamin E content oxidized by ferric chloride. Pappenheimer (8) has reported testis degeneration in guinea pigs subjected to chronic vitamin E deficiency.

Vitamin E deficiency and Muscular Dystrophy

In 1938 Evans and Burr (9) noted that young rats from females on a vitamin E low diet often showed a marked paralysis, especially of the hind quarters, about 20 days after birth. This paralysis extended to the body wall musculature.

In 1931 Goettsch and Pappenheimer (10) described a muscular dystrophy in rabbits and guinea pigs when fed a natural food diet treated with ferric chloride and cod liver oil. Ferric chloride had been proven destructive of vitamin E activity [Waddell and Steenbock (11)]. That vitamin E deficiency was the limiting factor in the diet which produced the dystrophy was demonstrated by the MacKenzies and McCollum (12). They showed that muscular dystrophy was producible by vitamin E deficient diets that contained no cod liver oil and was curable by a pure form of vitamin E (alpha-tocopherol).

TABLE 19

Effects of vitamin E deficiency in animals

(After Mason (4) 1944)

Animal type	Dystrophy of skeletal muscle	Changes in smooth muscle	Vascular system	Reproducti system & em
Rat:				
Fetus			Vascular stasis & hemorrhage	Resorpti
Nursing	Acute			
Adult	Chronic	Necrosis of uterus & seminal vesicles		
Mouse:				
Fetus				
Nursing	Slight			
Adult	Slight			
Guinea pig	Acute			
Rabbit	Acute			
Hamster	Acute			
Sheep	Acute*			
Goat	Acute*			
Kangaroo	Acute			
Dog	Acute			
Duckling	Acute			
Gosling		Gizzard necrosis		
Chick:				
Embryo		Lethal ring hemorrhage & death		
Pullet		Exudative diathesis, generalized edema & encephalomalacia		
Adults	Slight			
Guppy fish	Acute			

* Not proved to be prevented by pure tocopherol.

Quoting again from Mason (4):

> "The histo-pathological changes observed in muscular dystrophy are hyali waxy or Zenker's degeneration, usually associated with acute infections and fev There are loss of muscle striations, multiplication and irregular distribution of s colemma nuclei, and swelling of the sarcoplasm which in turn becomes structurel and vacuolated. There may be edema and inflammatory reactions in the intersti connective tissues and calcification of necrotic muscle fibers."

Why such dystrophy occurs from vitamin E deficiency and how vitan E acts to prevent it is still undetermined. It is certain that vitamin E is essential in muscle metabolism and Houchin (13) has suggested that tocopherols, probably in phosphorylated form and acting through some system of enzymes may inhibit or regulate the oxidative processes in muscle.

Antioxidant Properties of Vitamin E

Today many of the effects of vitamin E are traceable to its action as an antioxidant. In 1927 Simmonds, Becker and McCollum (14) and also Jones (15) showed that the inactivating effect of ferrous sulfate on vitamin A could be prevented by wheat germ oil (a rich source of vitamin E). A little later Mattill and Crawford (16) presented evidence that the protective effect of wheat germ oil was due to the presence of hydroxy groups which inhibit oxidation of fatty acids; in brief, the presence in wheat germ oil of an antioxidant.

Olcott and Mattill and coworkers (1) are largely responsible for clarification of the antioxidant action of vitamin E which is now established as one of its fundamental functions. Its oxidative destruction by rancid fats has explained the vitamin deficiency effects of diets containing such fats. It also explains the use of wheat germ oil or the tocopherols themselves as antioxidants in vitamin preparations where they protect oxygen sensitive vitamins such as vitamins A and C from loss of activity in storage.

The antioxidant action of tocopherols in animals got prominence from the work of Hickman and associates (17) confirmed by Lemley et al. (18) who showed that the biological response of vitamin A and carotene was greatly influenced by the presence or absence of vitamin E (see Table 20). In its absence vitamin A and carotene underwent oxidative destruction in both the gastrointestinal tract and in animal tissues. Further evidence of the protective action of vitamin E on vitamin A metabolism has been given by Clausen et al. These investigators made rats diabetic by injection of alloxan which quickly depletes body stores of vitamin A, even when rats are fed a normal supply of vitamin E. When they added as much as 0.5 mg. of extra vitamin E the loss of A was lessened and the life of the animals prolonged.

Such findings have increased the tendency to attribute deficiency effects of vitamin E to interference with the antioxidant action of the vitamin and to relate curative and preventive action to some manifestation of its antioxidant activity. However as Mason states:

> "The antioxidant function of the tocopherols, important as it may be, cannot represent the prime role of vitamin E in the animal body. The inability of other antioxidants to substitute for vitamin E, the inverse relationship between antioxidant and biological actions of alpha, beta and gamma tocopherols, lack of evidence that tocopherols retain their antioxidant activities after ingestion, and the manner in which changes unrelated to the phenolic hydroxyl group in the molecule alter bio-

logical activity of the tocopherols; all suggest a more fundamental and complex ro in metabolic processes."

TABLE 20

Effect of different quantities of vitamin E on the gain in weight accruing from fixe quantities of vitamin A

(After Hickman (17) 1943)

Amount and kinds of vitamin A	Amount of tocopherol supplement fed and gain in weight (grams) o male rats in 28 days						
	0 mg.	0.025 mg.	0.05 mg.	0.15 mg.	0.5 mg.	1.5 mg.	5.0 mg.
0.46 mcg. vitamin A	+8.6		+20.7	+28.8	+27.6	+31.7	
0.5 mcg. vitamin A acetate	−6.3	+4.0		+13.0		+25.0	
0.79 mcg. beta carotene	−22.0	−23.0	−21.0	+10.3	+29.3	+22.7	+12.

Comparison of tocopherols and other antioxidants on the growth efficiency of 0.8 mcg. of carotene

Supplements	Growth increase in 36 days in grams
	grams
None	2
0.15 mcg. mixed tocopherols	31
0.15 mcg. pure alpha tocopherol	27
0.15 mcg. pure beta tocopherol	30
0.15 mcg. pure gamma tocopherol	30
0.1 mg. ascorbic acid	7.5
1.0 mg. ascorbic acid	11.0
10.0 mg. ascorbic acid	20.0
0.5 mg. palmityl ascorbic	3.3
0.13 mg. hydroquinone	27.9
35 mg. Lauryl hydroquinone	30

N.B. There is also some evidence that ascorbic acid itself is protected in th body by vitamin E. The accumulation of ascorbic acid in the organs of guine pigs on low intake of ascorbic and variable amounts of vitamin E gave these results Animals given 6–10 mg. of ascorbic acid per kilo of body weight and 0.5–1.0 mg vitamin E per kilo of body weight showed liver increases in ascorbic acid of 19–22

Vitamin E deficiency and Cod Liver Oil

As early as 1926 Agduhr (19) called attention to serious morphologica and fundamental changes associated with dosages of cod liver oil. He note that these effects were mainly upon muscle and that the cat, dog, and ca were less resistant to its toxic effect than the mouse. (In 1942 Goettsc (20) showed that mice require much less vitamin E than rats.)

The relation of vitamin E deficiency to these effects was not at first sus pected; the search was for a toxic something in the cod liver oil. With the relation of vitamin E deficiency to muscular dystrophy established, attention turned to the determination of the way in which the cod liver oil inactivated the vitamin. That the effect might be due to rancidity of the oil appeared plausible. Madsen et al. (21) showed that hydrogenating the cod liver oil lessened its power to accelerate dystrophy symptoms and hydrogenation was known to be a means of protecting fats from rancidity. Other explanations were sought and investigated. At one time Morgulis and Spencer (22) held that two factors were involved, a water-soluble fraction of wheat germ and a fat-soluble fraction. Mattill and Golumbic (23) in 1942 summarized the situation at the time in the following statement: "Evidence is presented to show that no distinction need be made between a cod liver oil induced muscular dystrophy in rabbits and the nutritional muscular dystrophy produced by lack of vitamin E. None of the members of the vitamin B complex appears to be concerned with nutritional muscular dystrophy."

But the rancid oil theory failed to satisfy as an explanation of the cod liver oil effect. More light was thrown on the problem by study of vitamin E deficiency effects in chicks.

Vitamin E Deficiency in Chicks

In 1931 Goettsch and Pappenheimer (24) described a cerebellar disorder in chicks, apparently of nutritional origin. To quote from their report: "The disease was manifested only in birds receiving certain simplified diets; it was accompanied by characteristic symptoms and by uniform and well defined lesions of the cerebellum."

They called the disease encephalomalacia and described it as follows:

> "Growing chicks maintained on a diet consisting of milk powder, casein, starch, yeast, cod liver oil, salts and filter paper develop ataxia, tremors, retraction or twisting of the head, clonic spasms of the legs and stupor. These symptoms may appear suddenly, usually between the 18th and 25th day, and may end in death. If recovery takes place, the chicks may go on to normal development.
>
> Definite lesions are found in the cerebellum of the affected chicks. These consist of edema, necrosis, and hemorrhages. Hyaline thrombi are found in the capillaries in and about the degenerated areas."

It may be noted that the diet used was low in vitamin E and contained cod liver oil. Later these investigators reported that chicks could be protected from encephalomalacia by inclusion of certain edible oils (corn oil, cotton seed oil, peanut oil, soy bean oil) and that the protective factor was in the non-saponifiable fraction of the oils. Later still when synthetic vitamin E became available, Dam et al. (25) showed that it gave complete protection against the encephalomalacia.

But in 1939 Dam and Glavind (26) described another chick diseas They called this one exudative diathesis, and described it as follows:

> “A condition in which the plasma exudes from the capillaries, appears as massi accumulations of fluid in the subcutaneous tissue or as edema of the muscles and co nective tissue more generally. The affected tissue, especially the adipose tissue, usually reddish due to fine diffuse hemorrhage. Microscopically there is edema, slig extravasation of the red cells and migration of white cells into the affected tissue, ar a mild eosinophilia.”

The diets used by Goettsch and Pappenheimer and by Dam and Glavin produced one striking difference in the effect on chicks. With the form encephalomalacia was usual, exudative diathesis rare; with the Dam an Glavind diet the reverse was true.

As both diets contained cod liver oil Dam experimented to learn if th exudative diathesis could be attributed to an effect of the cod liver oi In 1943 he made this report:

> “The exudative diathesis characteristic of vitamin E deficiency in chicks readil occurs with diets containing 5 per cent of *fresh* cod liver oil, slightly rancid cod live oil, or a mixture of fresh and completely rancid cod liver oil; to a lesser extent b similar proportions of lard or fatty acids from linseed oil. On the other hand the symp toms do not occur if the diet contains a similar proportion of oleic acid or thoroughl rancid cod liver oil, or if the diet is rigidly free of fat.”

These findings cast doubt on the rancid cod liver oil theory but did em phasize that there was some relation of fats and fatty acids to the produc tion of the syndrome.

In a discussion of the problem in 1944 Dam (28) suggested a possibl relation between the exudative diathesis and encephalomalacia, namel that the latter might be due to capillary exudation into the brain tissu He also pointed out that the problem was complicated by the fact tha there is no complete parallelism between the factors which favor or counte act the two symptoms. If, for example, fats merely destroy vitamin E i the body, why do some favor exudation and some encephalomalacia, an why is dietary fat so essential for the development of symptoms but not i rabbits?

Some other findings that require explanation may be listed as follow

1. Purified diets containing no added fat rarely produce exudates an never produce encephalomalacia.

2. A certain protein/carbohydrate ratio is optimal for the appearan of exudates and further enhanced by those inorganic salts such as Na that tend to accumulate in the extra-cellular fluid. A high lard (30 per cen favors encephalomalacia more than exudates when the protein/carboh drate ratio is low.

3. Inositol (1.5 per cent) counteracts both symptoms; lipocaic (2 per cent) counteracts only exudative diathesis. Cholesterol (1 per cent) hastens exudates when the diet contains 5 per cent cod liver oil and a low salt content and counteracts encephalomalacia when the diet contains 30 per cent lard.

4. Ducklings on vitamin E deficient diet suffer neither the encephalomalacia nor exudative diathesis seen in rabbits; but chicks on such a diet do not develop muscular dystrophy.

5. When hog liver fat was fractioned that fraction containing the saturated fatty acids or oleic acid had no effect. The fraction containing unsaturated fatty acids produced encephalomalacia in 2 weeks.

So today, we attribute the action of fats, including cod liver oil, to their content of highly unsaturated fatty acids. Since peroxidases appear in fats about the time symptoms appear, it would indicate that the symptoms stem from tissue damage caused by abnormal oxidation products of the unsaturated fatty acids, an abnormal oxidation due to inadequacy of vitamin E.

Some Theories as to how Vitamin E operates

In line with the theory of tissue damage by oxidation products of unsaturated fatty acids Dam and Granados (30) reported that rats on an E deficient diet plus 20 per cent cod liver oil showed no exudative diathesis but a depigmentation of incisor enamel. Mason, Dam and Granados (30) demonstrated the deposition of an acid fast pigment in the adipose tissue of vitamin E deficient rats on a diet high in cod liver oil. Filer et al. (31) showed that lard would produce the same effect, that similar diets (but with linseed oil methyl esters) caused much pigment deposition in fat depots, and that arachidonic acid gave poor growth, anemia, and early death with atrophic adipose tissue. The pigment appears to be the ceroid pigment noted by Victor and Pappenheimer (32) in the livers of rats on a low protein, E free diet. According to Mason and Emmel (33) it is an abnormal metabolite having only transitory existence in rats on adequate vitamin E diets.

One of the accompaniments of muscular dystrophy is an increased excretion of creatine and consequently changed urinary creatine/creatinine ratio. In fact this ratio has been used in diagnosis of the disease and the study of its progress. Dam reported that vitamin E prolonged the life of rats on a low protein diet but did not attempt any explanation. Hove et al. (35) have suggested a possible relation of vitamin E to amino acid metabolism. They claim that stomach lesions produced in rats by low protein, essential fatty acids, low pyridoxine or simply reduced calories are curable by vitamin E.

Guided by the attempt to connect its antioxidant activity with muscular behavior, Houchin and Mattill (14) have postulated that vitamin E might function as the prosthetic group in some enzyme system which regulates muscular metabolism. Such a system, however, has yet to be demonstrated. Houchin has backed his viewpoint concerning the regulation by vitamin E of muscular oxidative action by a study of the oxygen uptake of dystrophic and normal muscle and the effect of vitamin E upon such action. Table 21 gives some of Houchins findings.

In 1942 Houchin and Mattill (36) claimed to get an increased rate of enzymatic oxidation of succinic acid by skeletal muscle of animals with muscular dystrophy. They found the oxygen consumption of normal rabbit muscle to be 1.4 cmm. O_2 per mg. of dry weight per hour; in rabbit dystrophic muscle, 3.12 cmm. of O_2 per mg. of dry weight per hour, together with a decrease in creatine and an increase in NaCl. Response to vitamin E administration was rapid. In hamsters a dose of 1 mg. of alpha-tocopherol brought oxygen consumption back to normal in 24 hours and administration of alpha tocopherol phosphate by ear vein reduced muscle oxygen consumption 33 per cent in one hour. This led Houchin (14) to study the ability of muscle to oxidize succinic acid *in vitro*. He found the oxidizing power of hamster dystrophic muscle 160 per cent above that of normal muscle. Addition of vitamin E *in vitro* produced marked diminution of that power.

TABLE 21

The in vitro effect of alpha-tocopherol phosphate on the oxygen uptake of muscle from vitamin E deficient and normal animal

(After Houchin and Mattill (36) 1942)

Animal used and condition	QO_2 averages control in Locke's solution	In Locke solution containing 5 mg. per cent alpha-tocopherol phosphate	Decrease
			per cent
E-deficient rabbit	2.54	1.45	41.3
E-deficient hamster	2.89	1.86	35.7
Normal rabbit	1.34	1.40	−4.3
Normal hamster	1.74	1.78	−0.8
Treated and deficient rabbit*	1.72	1.62	6.2

* Given a therapeutic dose of 25 mg. alpha-tocopherol acetate 48 hours previously.

Basinski and Hummel (37) have contradicted Houchin's succinic oxidase findings but their work does not preclude the possibility of water soluble tocopherols keeping succinic dehydrogenase in control. In a later paper they reported on the consumption of oxygen by skeletal muscle strips from normal rabbits receiving 15 mg of alpha-tocopherol acetate in olive oil daily and from E deficient rabbits. The oxygen consumption of the muscle of the E deficient rabbits was approximately twice that of the normal rabbit muscle but addition of 1 mg of di-sodium dl-alpha tocopherol phosphate had no significant effect on the oxygen consumption of either group.

Others have suggested a relationship between vitamin E and enzyme systems. Torda and Wolff (38) find such a relationship in the production by vitamin E of acetyl-choline in minced frog brains. Morgulis and Jacobi (39) claim that enhanced oxygen uptake in dystrophic muscle is caused by triphosphatase activity. Govier et al. (40) have reported that the digitonin-increased oxidation of lactate by E deficient guinea pig heart muscle homogenates could be prevented by alpha-tocopherol phosphate and that such an increase is not produced by normal heart tissue.

Is vitamin E concerned in creatine metabolism? On this point Mason (4) comments: "That creatinuria of nutritional dystrophy might be due to hydration of creatinine to creatine due to increased water content of the tissues is of interest, especially in view of the fact that alteration of normal water balance of tissues is a constant finding in biochemical and histological examination of tissues affected by vitamin E depletion."

But Mason has also said: "It is not surprising that none of the theories proposed so far to explain the physiological role of vitamin E has proven adequate."

The problem of exactly how vitamin E acts in correcting the various effects that follow its omission or inadequacy in diet has not yet been established. Table 19 shows how deficiency of vitamin E affects different species of experimental animals. For discussion of its relation to human nutrition see Section III.

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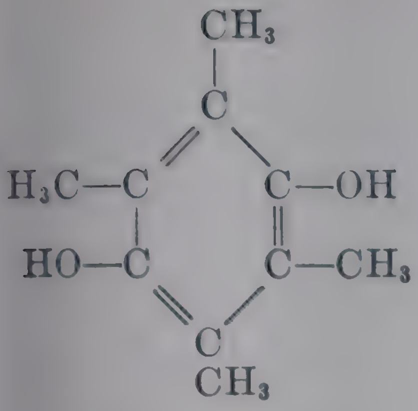
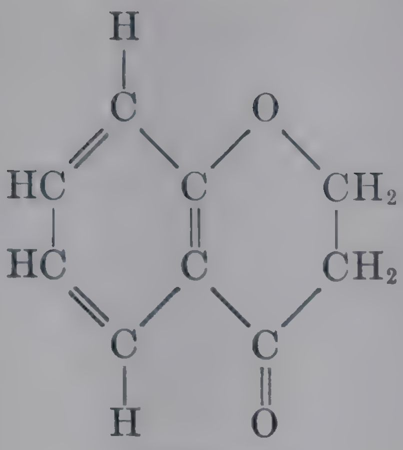
Section II. Forms and Chemistry of Vitamins E

At this writing four forms of vitamin E have been chemically identified and are known as alpha-, beta-, gamma-, and delta-tocopherol. The first three and their properties have been known for some time; delta-tocopherol was first described by Stern et al (1) in 1947.

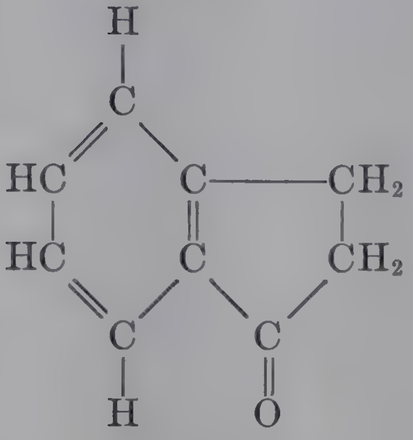
The following steps summarize the progress of chemical identification. Evans and Burr (2) in 1927 reported making potent concentrates from wheat germ oil. In 1934 Olcott and Mattill (3) obtained still more potent concentrates by high vacuum distillation and in 1935 Drummond et al. (4) also described concentrates of similar high potency.



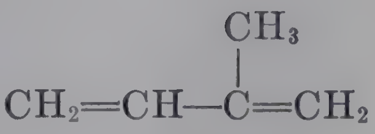
Durohydroquinone



Chroman

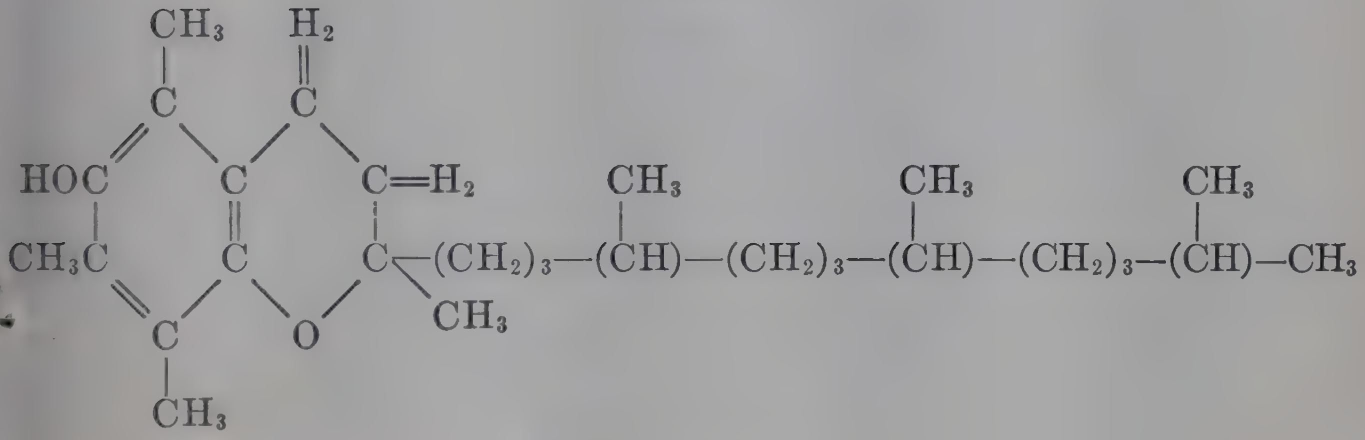


Coumaran

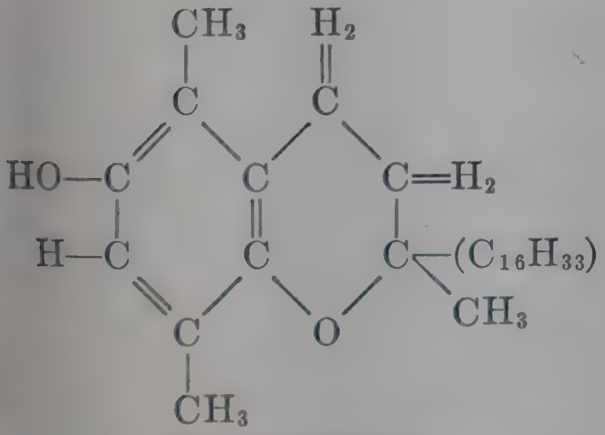


$CH_2{=}CH{-}\overset{CH_3}{\overset{|}{C}}{=}CH_2$

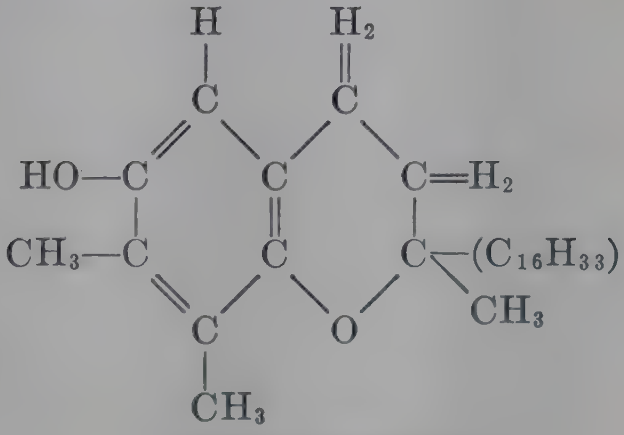
Isoprene



Alpha-Tocopherol

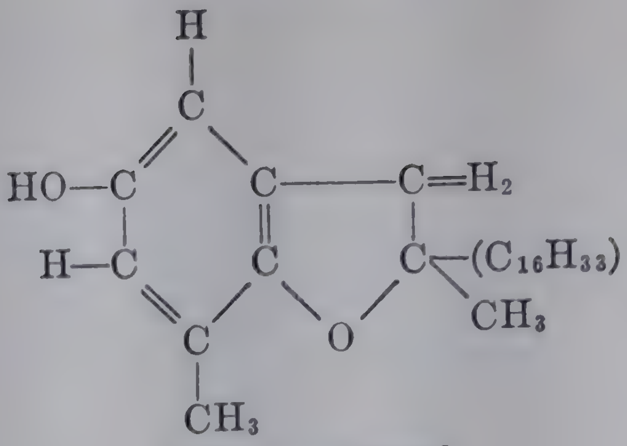


Beta-tocopherol



Gamma-tocopherol

FIG. 5



Delta-tocopherol
Tocopherol Structures
FIG. 5—*Continued*

In 1935 Olcott (5) and also Drummond et al. (4) demonstrated in the vitamin the presence of an easily esterified hydroxyl group. This made possible the formation and separation of crystalline allophanates of the vitamin. (Treatment with cyanic acid produces the allophanate). Employing this technique Evans and the Emersons (6) reported in 1936 the isolation of three different allophanates from wheat germ oil:

1. An allophanate of Beta-amyrin, melting point 250°C. but its regenerated alcohol showed no biological activity.
2. An allophanate with a melting point of 138°C. The alcohol regenerated from this showed slight biological activity.
3. An allophanate melting at 158–160°C. Its regenerated alcohol proved to be highly active biologically; 3 milligrams being adequate to prevent loss of fertility. They considered this the true vitamin E and established its chemical formula as $C_{29}H_{50}O_2$. They also reported an absorption band at 298 $\mu\mu$ and an $E^{1\%}_{1cm.}/ = 90$.

For reasons already explained in Section I (see page 60) they elected to call this alcohol tocopherol and in anticipation of other forms, alpha-tocopherol.

Discovery of other forms of the vitamin quickly followed. In 1937 Todd et al. (7) isolated an allophanate whose regenerated alcohol they called beta-tocopherol. It had a melting point of 143.5–144.5°C. The alcohol had an absorption band at 295 $\mu\mu$ and a 5 mg. dose was biologically effective.

In the same year the Emersons, Mohammad and Evans (8) reported isolation of gamma-tocopherol. As stated earlier no other forms were reported until 1947 (By Stern et al. (1)).

The structural makeup of these forms is shown in figure 5.

Determination of the Nucleus

In 1937 Fernholz (9) reported that an alpha-tocopherol obtained from cotton seed oil decomposed at 350°C. into a crystalline sublimate and an oily distillate. The crystalline fraction showed an empirical formula of $C_{10}H_{14}O_2$ and closely resembled durohydroquinone. The suggestion that the tocopherol might be a mono-ether of durohydroquinone was, however, shown to be untenable by a number of investigators (John (10), Bergel (11) and Karrer (12)) all of whom proposed a coumaran or chroman nucleus with a side chain of isoprene residues (see figure 5).

TABLE 22

Properties of forms of tocopherols

(After Hickman (14) 1943)

Tocopherols	Habit	Melting point	Mean fertility dose mg.	Absorption maxima μμ
Synthetic alpha (Merck)	Viscous oil		1.0	292
Synthetic beta (Merck)	Viscous oil		5.0	297
Synthetic gamma (Merck)	Viscous oil		20.0	300
Synthetic alpha-acetate (Hoffman-LaRoche)	Yellow oil		1.0*	286
Synthetic alpha-palmitate (Merck-Baxter)	White granules	33–38	2.0	286
Natural alpha (Baxter et al.)	Viscous oil		1.0	292
Natural beta (Baxter et al.)	Viscous oil		2.5	297
Natural gamma (Baxter et al.)	Viscous oil		12.0	298
Natural alpha acetate (Robeson)	Needles	26.5–27.5	1.0	286
Natural alpha acid succinate	Needles	76–77	1.0*	286
Natural alpha palmitate	Glossy laths	42–43	12.0*	286
Natural beta-azo-benzene carboxylate	Orange rosettes	69.5–70.5	8.0*	328
Natural gamma palmitate	Glossy laths	44–45	12.0*	286
Natural alpha allophanate	White granules	157–158		286
Natural beta-allophanate	Colorless laths	138–139		286
Natural gamma allophanate	White granules	136–138		286
Synthetic alpha-Ca-succinate	White solid	210–212	1.0*	?
Synthetic alpha phosphate (Na salt)	White powder		1.0*	?
Synthetic alpha tocopherol amine (HCl)	White solid	156–158	1.0*	?

* On basis of tocopherol content.

Actually, before the question of chroman or coumaran nucleus was settled, Karrer et al. (13) actually synthesized the vitamin by an almost quantitative condensation of trimethyl hydroquinone with phytyl bromide using zinc chloride as a catalyst. Except for optical activity the product had physical, chemical, and physiological properties of vitamin E. By 1938 it was settled that the nucleus was chroman attached to a phytyl side chain (see figure 5). Figure 5 also shows the modifications in structure which yield the 3 tocopherols known as alpha-, beta- and gamma-tocopherol. From these three forms a wide range of derivatives have been made. These forms and their chemical and physical properties have been tabulated by Hickman (14) and are shown in Table 22. Embree (15) has also tabulated the comparative biological properties of the first three forms as shown in Table 23.

TABLE 23

Relative biological potency of the tocopherols

(After Embree (15) 1946)

Forms	d-alpha tocol	dl-alpha tocol	d-beta tocol	dl-beta tocol	d-gamma tocol	dl-gamma tocol
For the rat:						
Antisterility	100	85	33	16	1	<1
Weight gain		100		25		19
Vitamin A protection	100		100		100	
For the rabbit:						
Creatinuria	100				15	
For the chick:						
Exudative diathesis		100				

Delta-tocopherol

Stern et al. (1) obtained delta-tocopherol from soy bean oil. They state that it forms 30 per cent of the tocopherols in soy bean oil, 5 per cent of those in wheat germ oil, and is present in cotton seed and peanut oils. It is the only mono-methyl tocopherol found to date (8 methyl-tocol).

Delta-tocopherol proved to be the most active antioxidant for vitamin A acetate and beta-carotene of the four tocopherols but it had only 1/100th of the biological activity of alpha-tocopherol by the Evans fertility test.

Delta-tocopherol has an absorption curve similar to the other three forms and Stern et al reported its $E^{1\%}_{1\text{cm.}} = 91.2$ @ 298 $\mu\mu$. Of all the forms the most active biologically is alpha-tocopherol.

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SECTION III: EVALUATION OF VITAMIN E FORMS FOR HUMAN AND ANIMAL NUTRITION

The use of vitamin E in human therapy has been disappointing to date and the American Medical Association's Council on Pharmacy and Chemistry (1) summarizes the situation as follows:

> "For nearly two decades it has been known that vitamin E must be included in the diet of the rat to insure successful reproduction. There are at least three naturally occurring compounds which have vitamin E activity: alpha-, beta- and gamma-tocopherol. There have been comparatively few clinical studies dealing with the role of vitamin E in human physiology and they have not led to very definite conclusions. There seems to be agreement that the vitamin is of no value in the treatment of sterility. There are indications that it may be of value in the treatment of habitual abortion but further studies are necessary to clarify the picture.
>
> Recently there has been renewed interest with respect to vitamin E owing to reports that administration of alpha-tocopherol and other preparations of vitamin E have produced beneficial results in the treatment of some cases of degenerative diseases such as amyotrophic lateral sclerosis. This is not substantiated in any way by recent clinical evidence."

One of the difficulties in evaluating vitamin E in human physiology is that the findings in laboratory animals are not transferable to other species (see table 19). Even in a given species, such as the chick, changes in dietary ingredients other than vitamin E content change the form of the pathology; encephalomalacia occurs with one diet, exudative diathesis with another diet.

Mason (2) comments on the problem as follows:

> "The difficulties in statistical treatment of results in the face of inadequate control material, the problem of properly evaluating the effect of other therapeutic measures instigated in connection with vitamin E therapy, the extensive use of wheat germ oil or concentrates of the latter rather than the pure vitamin in earlier studies, and the difficulties of explaining an assumed deficiency state in the face of presumably adequate diets, all contribute to the perplexity of the problem and the difficulties of evaluating the clinical usefulness of the vitamin."

A typical example of the difficulty of evaluating clinical reports is found in two reports on the value of vitamin E in treating purpura. Skelton et al. (3) reported cure by vitamin E of a true purpura in dogs produced by injections of stilbestrol; also that synthetic alpha-tocopherol acetate in daily doses of 200–400 mg. daily was effective in human purpura. On the other hand Rightsmeier et al. (4) were totally unable to confirm their findings.

With the synthetic forms of vitamin E now available the future should show definite progress in elucidating the role of vitamin E in human and animal physiology or as Embree (5) puts it: "The place of vitamin E in human and farm nutrition is a problem still requiring the best attention of scientific workers."

Effect on Human Fertility: In the quotation from the Council of Pharmacy and Chemistry was the statement: "*There are indications that it may be of value in the treatment of habitual abortion.*"

The records of cases reported up to 1938 were studied by a referee of the Council (6). Of his findings (see Table 24) he concludes as follows:

> "The claim that vitamin E is of value in the prevention of habitual abortion cannot be accepted because of lack of *convincing* clinical evidence. The diagnosis of habitual abortion in many of the published reports is open to question; the great variation in dosage of vitamin E and the lack of evidence that the preparations used were active make it difficult to attribute any effects claimed for it to the vitamin. Moreover, the expectancy of spontaneous cure in cases of so-called habitual abortion has not been accurately established. The published results of the treatment of habitual abortion with vitamin E are sufficiently encouraging to justify further clinical experiment. Such experiments are justified only if preparations of vitamin E of known activity are used and if adequate diagnosis and clinical control can be established."

The evidence of value of vitamin E in treatment of disorders of menstruation, the toxemia of pregnancy, faulty lactation and vaginal conditions naturally led to the hope that some human muscle dystrophies might yield to vitamin E therapy. Results to date, however, have been mostly disappointing.

Shank et al. (12) reported a study of 40 cases of progressive muscular dystrophy but had little success with vitamin E therapy, and failed to confirm the earlier hopeful observations of Bicknell (13). Hoagland et al. (14) reported in 1945 a detailed study of 6 boys with progressive muscle dystrophy and compared findings with 5 normal boys. They did conclude that because of its constancy the diminished excretion of creatinine in dystrophy is a more specific diagnostic sign than excessive creatine excretion. They point out that many other diseases and physiological states in normal individuals can lead to excessive output of creatine.

Minot and Frank (15) supplemented their clinical experiments with quantitative determinations of the concentration of tocopherol in the blood serum of boys with pseudo-hypertrophic muscle dystrophy and in normal boys of the same age. The patients were treated with a considerable variety of preparations containing vitamin E as well as with pure alpha-tocopherol.

They report that the tocopherol content of the blood serum of untreated patients with muscular dystrophy ranged from 0.73–1.28 mg. per 100 ml.; in normal children the range was 0.64–1.12. Following the administration of vitamin E to the dystrophy group there was an increase of 20–30 per cent in the blood level of vitamin E *but no significant clinical improvement and no change in degree of creatinuria.* They point out, however, that there may exist some interference with intermediate steps in the utilization of alpha-tocopherol which blocks the effectiveness of the vitamin in relieving the muscular dystrophy.

TABLE 24

(From J. Amer. Med., Assoc., 114: 2214, 1940)

A. Use of wheat germ oil in prevention of repeated abortion

Author	Dates	Cases	Successful	Daily doses
Vogt-Möller	1931	2	2	5 cc. wheat germ oil
Vogt-Möller	1934	20	17	3 gm. "Fertilan"
Vogt-Möller	1936	52	38	3 gm. "Fertilan"
Juhasz-Shaffer	1933	2	2	?
Currie	1935	37	34	3 minims (0.2 cc.) wheat germ oil
Watson & Tow	1936	47	34	1–6 cc. wheat germ oil
Bishop	1937	2	1	3 capsules "Fertilol"
Cromer	1938	4	2	?
Malpas	1938	9	2	?
Totals		175	132	

B. Relationship between number of previous abortions and results of treatment with wheat germ oil

Number of previous abortions	Number of cases	Results successful	Per cent of successful results
1	14	10	71
2	71	51	72
3	32	24	75
4	31	26	84
5	21	16	76
6	2	2	100
Not stated	3	3	100
Totals	174	132	75

Some positive results have been claimed for vitamin E therapy in muscular dystrophy of certain types. Two are of particular interest in suggesting combination with other vitamin material for effective action.

In 1940 Stone (16) reported treatment with wheat germ oil of 5 patients with muscular dystrophy; one with muscular atrophy following anterior poliomyelitis; one with muscle atrophy after an attack of multiple neuritis. He found: "Definite improvement was obtained in all cases with muscular dystrophy, the improvement being manifested in gain in muscle strength, the disappearance of fatigue and muscle pain on slight exertion, change in muscle texture, and displacement of dystrophic musculature by normally contracting muscle." Also: "*The addition of vitamin B complex to vitamin E appeared to increase the therapeutic efficaciousness of the latter.*"

In 1945 Milhorat and Bartels (17) reported that tocopherol *plus the B-Complex vitamin inositol* decreased the creatinuria in dystrophic patients. They studied 15 patients with progressive muscular dystrophy resembling that in vitamin E deficient animals. Recalling that in animals, when tocopherol is given orally, but *not* parenterally, creatinuria is promptly reduced and muscle function restored they surmised that some change of the vitamin in the gastro-intestinal tract might be necessary for its utilization. The change suggested was a condensation with inositol and in proof of their viewpoint a mono-ether of inositol and tocopherol was prepared and found effective with their patients. When instead of the mono-ether, inositol itself and tocopherol were administered simultaneously they were only 1/8th to 1/30th as effective as the mono-ether but more effective than tocopherol alone. Incubation of the mixed vitamins with an extract of hog stomach and duodenum increased effect. The cases of Milhorat and Bartels were types of fibrositis and Steinberg (18) reported beneficial effect of vitamin E on 30 cases of human fibrositis in 1942.

Milhorat and Bartels offer the following hypothesis to interpret their findings, viz, that tocopherol forms a condensation product with inositol in the gastro-intestinal tract. The ability to synthesize this condensation product has been lost in hereditary muscle dystrophy. Patients in whom the disease is mild synthesize enough condensation product when large amounts of both vitamins are given but in rapidly progressing cases the condensation product itself is required. In a more recent report they claim that a derivative of tocopherol formed by a consequence of oxidation and reduction when given by mouth to human subjects with muscular dystrophy brings about a definite drop in the excretion of creatin. The new form of the vitamin is D-para-alpha-tocohydroquinone.

Vitamin E in Heart Disease

Mason (2) states that he has frequently observed in the heart muscle of rats subjected to prolonged E depletion, lesions resembling those seen in striated muscles. Vogelsang and Shute (19) have attempted to give vitamin

E a place in the management of heart disease. They claim that administration of large doses (200–600 mg. ephinal) of vitamin E had no apparent effect on normal hearts but on patients with congestive heart disease and the anginal syndrome the effect was markedly beneficial; it increased exercise tolerance, diminished or abolished anginal pain during the period of administration and had a pronounced diuretic effect. They attribute the effect of vitamin E on coronary pain to direct action on the coronary vessels or to some action on the metabolism of the heart muscle.

Baer (24) in a carefully controlled study failed to get confirmation of the value of vitamin E in heart cases and the claims of Vogelsang and Shutes need further investigation.

Dietary Requirements for Vitamin E

Because of its wide distribution in natural foods the chance of vitamin E deficiency in most human diets is small. In their study of American foods, Quaife and Harris (20) showed that these foods furnish an average of 15 mg. of d-alpha-tocopherol and an equal quantity of a mixture of the beta-, gamma-, and delta-forms per person per day. But they point out that diets low in vegetable oil products might fall below this figure. Hickman and Harris (21) estimate the normal adult human requirement as about 30 mg. of mixed tocopherols per day. The problem of estimating human needs is however complicated by the fact that the needs may vary at different age periods. Rats have been shown to have a critical requirement during the third week of life.

If the use of vitamin E in the human body parallels that in animals its antioxidant action makes it of value in the utilization of vitamin A and carotene and it may play an important role in oxidative metabolism or in the behavior of fats.

Vitamin E and Domestic Animals

The Committee on Animal Nutrition of the National Research Council has published data on vitamin E requirements for cattle, poultry, sheep and swine (22). In poultry, a lack of vitamin E in the ration of growing chicks results in encephalomalacia or exudative diathesis. According to Mason (2) 1.1 mg. of alpha tocopherol per kilo of body weight is necessary to prevent the encephalomalacia and something less than 3 mg. per kilo of body weight to prevent the exudative diathesis.

In many chicks deficiency results in subcutaneous edema and edema of the heart and pericardium.

In mature fowls prolonged vitamin E deficiency results in sterility in the male and reproductive failure in the female. Egg production is apparently not influenced by vitamin E but hatchability of the eggs is definitely reduced in E deficiency.

In poults vitamin E deficiency produces nutritional myopathy characterized by lesions in the muscular walls of the gizzard. They appear as circumscribed gray areas resembling scar tissue.

For cattle the need for vitamin E has not been demonstrated nor is there evidence of any rumen synthesis. Harris et al (25) reported that feeding 1 mg. of mixed tocopherols to cows daily increased the butter fat content of the milk but Gulickson et al (26) and Phillipps et al (27) were unable to get this effect.

For sheep a "stiff lamb disease" has been reported which is a specific muscular stiffness occurring in lambs varying from a few days to a few weeks in age. Willaman (24) suggests that it may be due to lack of vitamin E in the ration.

For swine vitamin E is necessary for normal reproduction but its necessity in the diet of growing pigs has not been shown to date.

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CHAPTER V. VITAMIN K

SECTION I. FUNCTIONS OF VITAMINS K

In 1929 Dam (1) described a hemorrhagic disease of chicks characterized by slow clotting of the blood. In 1931 McFarlane et al. (2) in Canada reported that chicks fed on extracted fish meal suffered a high mortality and extensive bleeding from small wounds; that their blood failed to clot even after hours of standing.

There are various theories on blood clotting but general agreement exists that two steps are involved. These steps may be described as follows:

1. Prothrombin present in the circulating blood plus thromboplastin liberated from the blood platelets plus ionized calcium react to form an enzyme called thrombin.
2. The action of thrombin on a soluble protein in the blood called fibrinogen causes it to coagulate and form a clot.

Failure of supply of any one of these reactants would be sufficient to prevent blood clotting. In the cases described by Dam and MacFarlane it was the prothrombin that was lacking in adequate quantity. In 1935 Dam (3) attributed the chick reactions as owing to the lack in the diet of a factor necessary to prothrombin production. He called it the "Koagulationsvitamin" and hence its present designation as vitamin K.

Prothrombin Formation

The site of prothrombin formation is the liver. Animals from whom most of the liver has been removed show a rapid decline in the blood prothrombin level. Furthermore animals deprived of liver tissue fail to produce prothrombin even when given large doses of vitamin K. Again, damage to the liver that interferes with utilization of the vitamin is not corrected even when large amounts of K are brought to it in the blood stream.

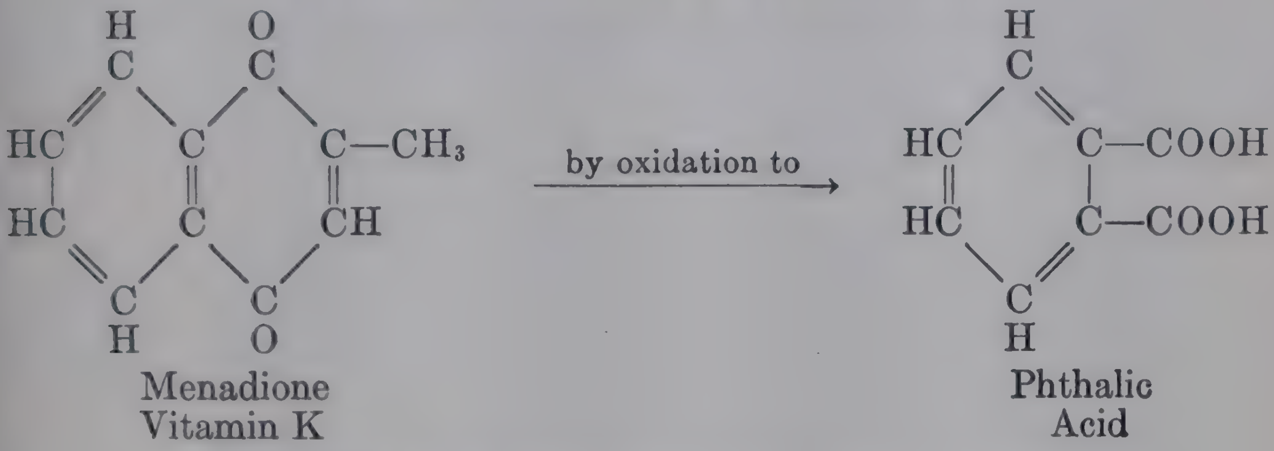
The exact manner in which vitamin K acts upon the liver tissue to produce a supply of prothrombin is still unknown. It does not form any part of the prothrombin molecule. If it were a part of the prothrombin molecule, which is a protein, it would logically be liberated in digestion but Dam (4) fed large doses of prothrombin to vitamin K deficient chicks with no effect on clotting ability.

Still further evidence that vitamin K acts upon the liver and not by combination with blood constituents is found in the fact that addition of K to blood drawn from a vitamin K deficient chick does nothing to restore its clotting potency but when the vitamin is fed to a vitamin K deficient chick its blood recovers normal clotting time in a few hours.

Theories of How Vitamin K Acts

McCawley and Gurchot (5) have made speculation on its possible action. They noted that the redox potential of vitamin K is close to that of para-benzoquinone. Para-benzoquinone has been shown to inhibit the proteolytic action of the cathepsin of the liver. Since prothrombin is a protein if vitamin K acted like para-benzoquinone and prevented the lytic action of cathepsin on the prothrombin the net results would be increase in the liver output of prothrombin. This is still only a speculation.

A. Relation of vitamin K to Phthalic Acid.



B. Dicumarol (3,3′ methylene-bis(4-hydroxy coumarin))

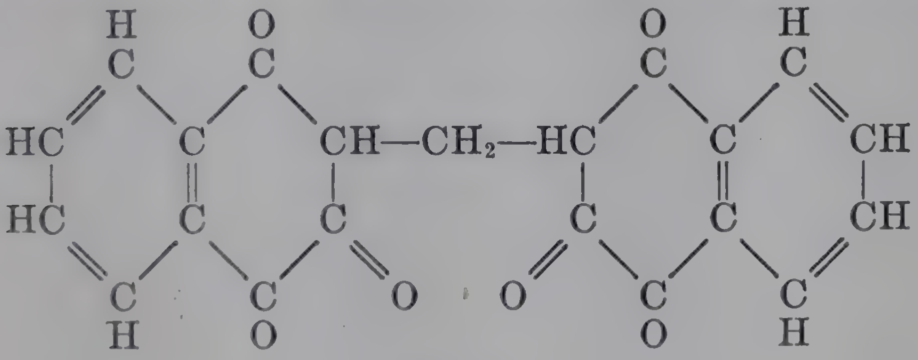


FIG. 6

Several investigators (6, 7, 8), have suggested that vitamin K activity is caused by final conversion into phthalic acid (see fig. 6) and it is the phthalic acid that actually functions. Evidence for this consists, according to Blumberg and Arnold (9), largely of the following:

1. That the highly active synthetic vitamin K (Menadione) sulfonate can be transformed into phthalic acid under conditions sufficiently mild to be duplicated in the body.

2. Experimental evidence of a slight anti-hemorrhagic activity of phthalic acid and a much higher activity of di-ethyl phthalate.

This viewpoint has been challenged. Dam (10) administered to young

(300 gm.) K-deficient chicks 100 mg. of phthalic acid and 23 mg. of diethyl phthalate and got no evidence of vitamin K activity. Karrer and Koller (11) reported that neither intravenous sodium phthalate nor oral diethyl phthalate was able to increase prothrombin time in a jaundiced subject who responded readily to an intravenous injection of menadione phosphate.

When tested by oral administration to baby chicks by Blumberg and Arnold (9), dipropyl, di-isopropyl, or di-butyl phthalate at a level of 10 mg. per chick or by diethyl phthalate in doses as high as 100 mg. per chick manifested no K activity. Neither did di-ethyl phthalate exhibit any anti-vitamin K activity such as has been reported for indanione derivatives.

As MacCorquodale (12) puts it: "The fundamental question of the exact relation of vitamin K to prothrombin formation still stands as a challenge to physiologists and biochemists."

The Basic Function of Vitamins K

To prevent hemorrhage by maintaining the normal clotting power of the blood through stimulation of prothrombin production in the liver is the basic function of the various forms of vitamin K. It is through operation of this particular function that vitamin K accomplishes most of its effects on animal physiology and finds utility in therapy.

There are, however, other effects which have been attributed to specific chemical groups in the vitamin K molecule.

Possible action on Hypertension

In 1944 Schwarz and Ziegler (13) reported that the parenteral administration of vitamin K lowered the blood pressure in hypertensive rats. The explanation suggested is that the quinone group acts on pressor amines, an action preventing their production of the hypertension. Soloway and Oster (14) showed that four out of ten quinones tested, when given orally or subcutaneously to hypertensive rats, lowered the blood pressure; three of the four were para-quinones, the fourth a diorthoquinone. They did not affect the blood pressure of normal rats. Grollman and Harrison (15) confirmed this and also showed that certain fish body and liver oils contained a substance they believed to be a quinone produced by oxidation of the marine oils which acted in the same way, i.e., reduced blood pressure in hypertensive rats.

It has been suggested that in an ischemic kidney, amino acids can be decarboxylated without being deaminized (16). Bing and Zucker (17) produced acute renal hypertension in cats by injection of l-dihydroxy-phenylamine into the partially or completely ischemic kidney. The explanation suggested that the amine was converted into hydroxytyramine, a pressor amine. Furthermore, this pressor amine does not accumulate when Bing and Zucker's amine is injected into the kidney under normal conditions of blood flow, probably because in that case the hydroxytyramine is destroyed by an amine oxidase.

If the quinone group in vitamin K acts to inhibit the action of pressor amines it is acting as a drug.

The theory was tested further by Moss and Wakerlin (18) on dogs made hypertensive by the Goldblatt (19) technique. They fed vitamin K orally to four dogs and intramuscularly to another in 60 mg. doses for a period of 6 months. An additional dog got 30 mg. daily for 3 months. They got no significant results with vitamin K, but on the other hand 6 lots of marine oil concentrates produced excellent results, 2 slight effects and 3 no effect. If the effective agent in the marine oil concentrates was a quinone as suggested by Grollman, the effect of K in favorable tests may have been caused by liberation of the quinone.

Possible Effect of Vitamin K on Tooth Decay

Burrill et al. (20) reported in 1945 a study of the effect of vitamin K incorporated in chewing gum on the incidence of tooth decay in human subjects. They used 119 subjects divided into two groups. One group got the gum containing in the sugar coating of the pellet 0.75 mg. of the sodium bisulfite product of menadione. The control group got the same type of gum without the vitamin K. The procedure was for the subjects to chew one pellet of gum for ten minutes or more after each meal. They report that after 18 months, examination of the teeth showed reduction in caries incidence in the K-gum group that was significant. The calculations of the authors have been questioned and the difference held not as great as claimed. Another test reported in 1946 failed to confirm the findings reported by Burrill et al. In this test (21) 16 men chewed a vitamin K impregnated gum for ten minutes after consuming any food or liquid. Fourteen men did the same with non-impregnated gum. In this series no difference which could be called statistically significant was found between the two groups.

If there is an effect of the vitamin K on tooth decay, it has been suggested that it might be due to antibacterial action of its quinone component, again a drug action of a vitamin K component.

Relation of Vitamin K to Abortion

To vitamin K has been attributed some effect in the prevention of abortion. Moore et al. (22) noted that rabbits fed a diet deficient in vitamin K invariably aborted during the first and second trimesters of pregnancy. They developed vaginal bleeding but no other hemorrhagic symptoms. King (23) has reported that in cases of human abortion, prothrombin time and vitamin C levels are lower than in controlled determinations in normal pregnancies and in persons in good health. In maintaining normal prothrombin content of the blood vitamin K may have an indirect action in the prevention of abortion.

Relation of Vitamin K to Salicylates

Link et al. (24) in 1943 demonstrated that salicylic acid can induce prothrombopenia in rats and that such prothrombopenia can be prevented by dosage with vitamin K. Following this observation further investigation (25, 26), showed that the observations made from the rat study also apply to man. Shapiro et al. (27) found it possible to prevent the prothrombopenia induced by 6 grams of acetylsalcylic acid given for one day by the intramuscular administration of one mg. of menadione. Shapiro (27) gives results of a more detailed study of the problem and concludes as follows:

> "Approximately 1 milligram of menadione will counteract the throbopenia-inducing action of one gram of acetylsalicylic acid. When factors such as fever, toxemia and limited nutritional intake complicate the situation, adjuvants such as ascorbic acid may be needed also. This can be determined by serial estimations of prothrombin time."

Fitzgerald and Webster (28) have also shown that barbiturates given as analgesics also lower blood prothrombin content and again, can be counteracted by vitamin K.

Dicumarol

The study by Link et al. (24) of the causal factor for hemorrhagic spoiled sweet clover disease led to the study of the action of vitamin K on salicylate-induced prothrombopenia. They found in the spoiled sweet clover a thrombopenia-inducing substance to which has been given the name "dicumarol". Dicumarol produces its effect only *in vivo* and there is a latent period before the prothrombopenia becomes detectable. The thesis is that the delayed action is due to metabolism of the dicumarol (methylene-bis-coumarin) to a derivative which is or from which the effective agent is liberated. Quantitative degradation of dicumarol to two mols of salicylic acid stimulated the chemists to the investigation of salicylate action.

Some twenty years ago in certain regions cattle, after consumption of spoiled sweet clover, developed a hemorrhagic condition due to marked reduction in prothrombin content of the blood. In 1941 Campbell and Link (29) reported recovery of a crystalline product having anti-blood coagulation potency. The product turned out to be 3-3 methylene bis (4 hydroxy coumarin). This product, when fed to rats, rabbits, guinea pigs or dogs, induced severe hypoprothrombinenia and ultimately fatal hemorrhages.

This effect could be counteracted by vitamin K and in some animals the K effect was enhanced by a supplement of ascorbic acid. The product now known as dicumarol has been studied as a possible agent for reduction of blood clotting under conditions such as coronary thrombosis. Also since it is an anti-vitamin K factor it has been hoped that study of its action might throw light on the physiological action of vitamin K itself.

Other Functions Attributed to Vitamin K

Fieser and associates (30) suggest that cancer-inducing hydrocarbons are known to be detoxified by interaction with suitable di-sulfide compounds such as prothrombin and it is conceivable that one of the normal functions of prothrombin may be the protection of the body from carcinogens. If that is the case vitamin K, by maintaining top level of prothrombin in the blood could possibly help to combat cancer formation. Fieser reports that rabbits, fed cancer causing substances, failed to develop cancer and excreted from the kidneys what appeared to be detoxified derivatives of the carcinogen.

In a study of factors aiding lactation in mice, Fenton et al. (31) showed that the addition to a synthetic diet of certain vitamins improved lactation. The supplements that were effective were biotin, cystine and vitamin K.

Chamoro (32) claims that daily administration of 20–30 mg. of menadione for a period of two weeks to immature rabbits stimulated growth of the mammae, the vagina, and the uterus.

Lecoq et al. (33) report that vitamins A, B_6, and K counteract the effect of adrenaline; vitamins P and C increase adrenaline action as determined by chronaximetric analysis.

Vitamin K is also said to activate trypsin (34).

The use of vitamin K in therapy is primarily utilization of its basic function, viz. stimulation of liver production of prothrombin. For discussion of the clinical uses in animal and human physiology see Section III.

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Section II. Forms and Chemistry of Vitamins K

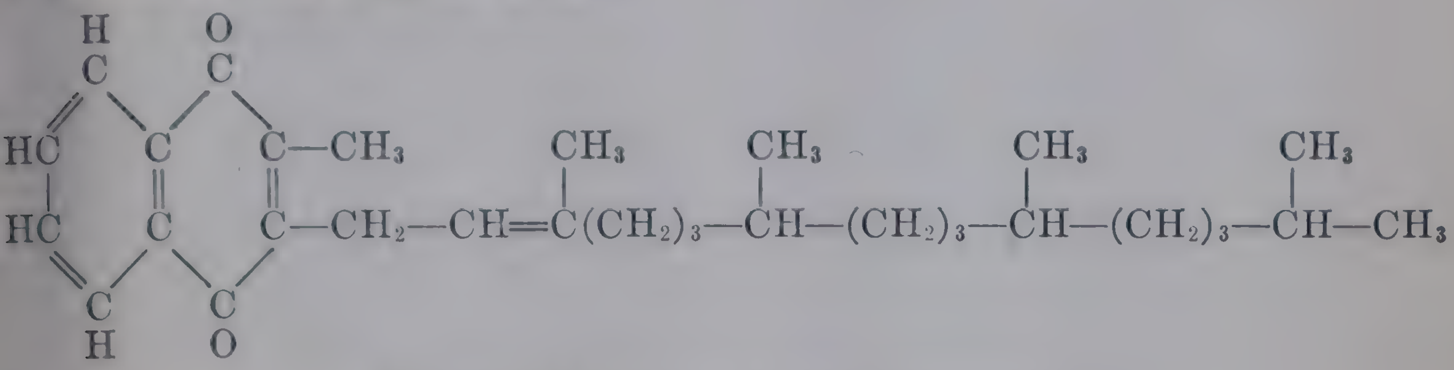
Two forms of vitamin K known respectively as vitamins K_1 and K_2 occur naturally. In addition there have been produced a considerable variety of synthetic compounds possessing more or less vitamin K activity. Of these synthetic compounds one, 2 methyl-1,4 naphthoquinone proved to be the most potent and is known today as "menadione".

The chemical structure of these forms is shown in figure 7.

The Natural Vitamins K

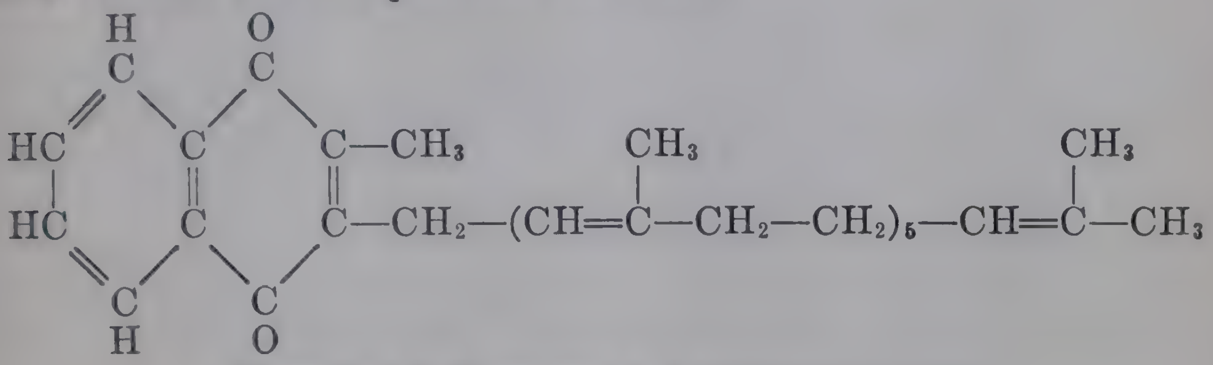
Dam (1) first isolated the natural vitamin from hog liver fat and showed it to be present in the sterol-free, nonsaponifiable fraction. In the same year (1935) both Dam (2) and Almquist and Stokstad (3) showed it present in high concentration in alfalfa.

1. Natural Vitamin K_1 from alfalfa.

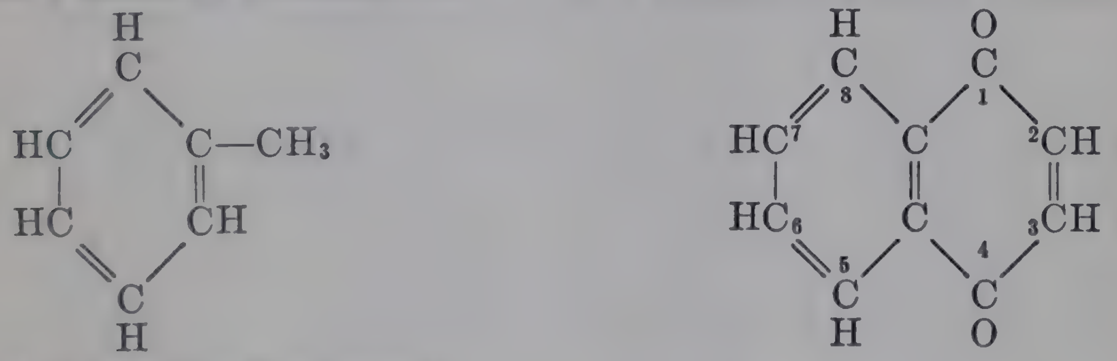


(2 methyl-3 phytyl-1,4 naphthoquinone)

2. Natural Vitamin K_2 from putrified fish meal.



(2 methyl-3 difarnesyl-1,4-naphthoquinone)



3. Synthetic Vitamin K (Menadione).

(2 methyl-1,4 naphthoquinone)

4. The naphthoquinone Nucleus.

FIG. 7. FORMS OF VITAMINS K

The pure vitamin K (now known as vitamin K_1) was first isolated and chemically identified (4, 5), in 1939 from alfalfa. Later Dam et al. (6) showed that this form of vitamin was found in the chloroplast of green leaves and was intimately related to their chlorophyll content. They found that leaves grown in the dark lacked both chlorophyll and vitamin K; that plants which can form chlorophyll in the dark such as spruces and pines also formed vitamin K in the dark. It is now known that leafy green plants are all good sources of vitamin K1.

Vitamin K_2 was isolated from putrified fish meal by McKee et al. (5) in 1929. Almquist and Stokstad (7) had reported the formation of a vitamin K by bacteria in 1936 and in 1938 Almquist et al. (8) showed this ability to be shared by a variety of bacterial organisms. In 1939 also McKee et al. (9) reported finding vitamin K active material in the feces of the horse, cow, sheep, hog, and man. As stated above McKee et al. (5) actually isolated the bacterially formed vitamin K_2 from spoiled fish meal in 1939. It shared with vitamin K_1 the naphthoquinone nucleus but with a different side chain attached to position 3 (see figure 7).

Chemical characteristics of Natural Vitamins K_1 and K_2

Vitamin K_1 as isolated from alfalfa is a light yellow oil at ordinary temperatures. It forms crystals at low temperatures but these crystals melt at −20°C. The oil has a specific gravity of 0.967 and a refractive index at 25°C of 1.5250. It has a lower molecular weight than vitamin K_1 (Empirical formula of K_1 is $C_{31}H_{46}O_2$; of K_2 is $C_{41}H_{56}O_2$).

K_1 is 2 methyl-1,4 naphthoquinone nucleus with a phytyl group attached in position 3. This structure has been confirmed by actual synthesis of vitamin K_1.

When used in therapy the dose for human beings is 4–10 mg. by mouth. It may be given intravenously in dextrose solution to adults in 10 mg. doses and to infants in 0.25 mg. doses.

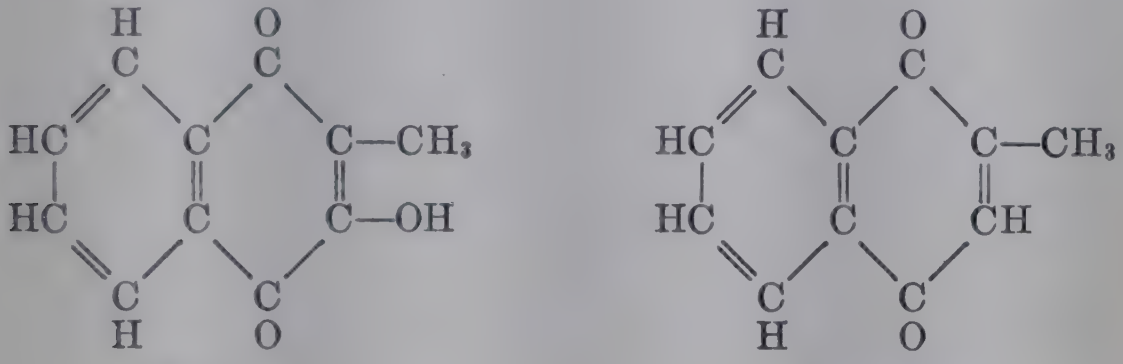
As might be expected from the difference in molecular weights vitamin K_2 is a light yellow crystalline solid melting at 53.5–54.5°C. Its structural formula is similar to K_1 as to the naphthoquinone nucleus but with a different side chain (difarnesil instead of phytyl) attached in position 3. This structure has yet to be confirmed by synthesis.

Both of the natural vitamins K are insoluble in water but soluble in fats and fat solvents.

Fowls experience difficulty in absorbing the vitamin K_2 from the intestine and hence on a K-deficient diet they develop the hemorrhagic disease. Human beings, rats and dogs readily absorb the K_2 formed by bacteria in their intestines and do not succumb so readily when put on a K-free diet. Man for example, normally obtains his requirement of vitamin K from the action of his intestinal bacteria without need of dietary supplementation. If however bacterial synthesis is inhibited, by the use of sulfa drugs, the disease will develop unless the diet is supplemented with some form of vitamins K.

Synthetic Vitamins K

A report by Almquist and Klose (10) that phthiocol (first isolated from tubercle bacilli by Anderson and Newman (11) in 1933) would prevent the hemorrhagic disease in chicks kept on a K-free diet initiated the development of synthetic forms of vitamin K. Figure 8 shows how closely phthiocol is related to 2 methyl-1,4 naphthoquinone. Its activity resulted in the discovery of the much greater activity of the latter compound now known as "menadione". Thayer et al. (12) claimed menadione to have twice the vitamin K activity of natural vitamin K_1 and phthiocol has only 1/500th of the activity of menadione.



Phthiacol

Menadione

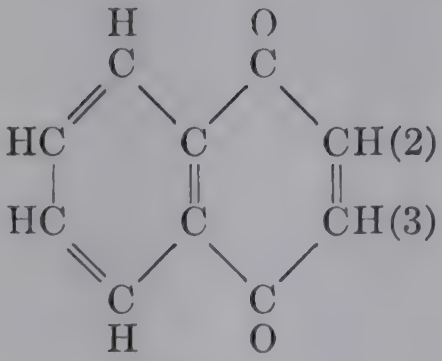
Fig. 8 Comparison of Phthiacol and Menadione

Menadione is a slightly yellowish, crystalline material rapidly destroyed by alkalies, sunlight and oxidizing agents and by strong acids. It is stable to heat and to contact with air and moisture. It may be prepared by oxidizing 2 methyl naphthalene with chromic acid. Its crystals melt at 105–107°C.

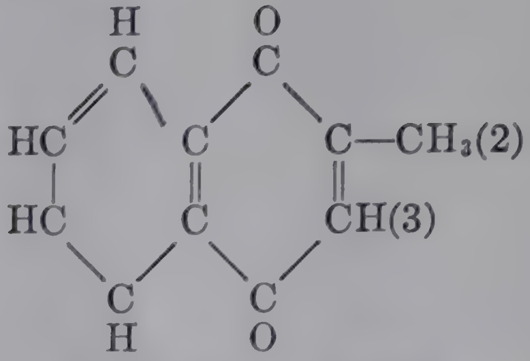
Like natural vitamin K_1 and K_2 it is insoluble in water but soluble in fats and fat solvents. For that reason, when given orally, it is better absorbed in the presence of bile salts which are usually administered with it.

There has been extensive study of the derivatives of naphthoquinone and it was found possible to produce water-soluble derivatives sufficiently active to allow intravenous injections of the vitamin. One method used is to combine hydroquinones with dibasic or tribasic acids and to employ their sodium salts.

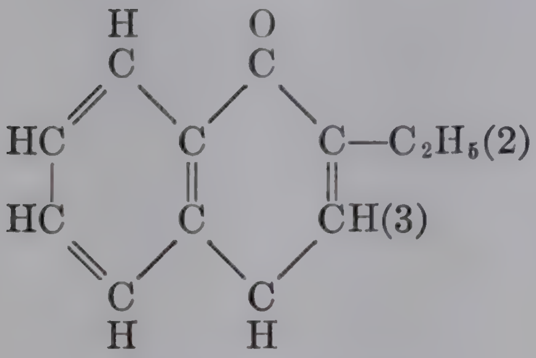
While there are a number of naphthoquinone derivatives with vitamin K activity the relatively high potency of menadione has made it the most commonly used form in vitamin K therapy. It is supplied today by drug



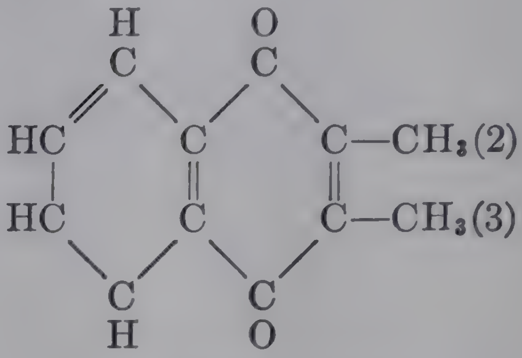
(1) Inactive



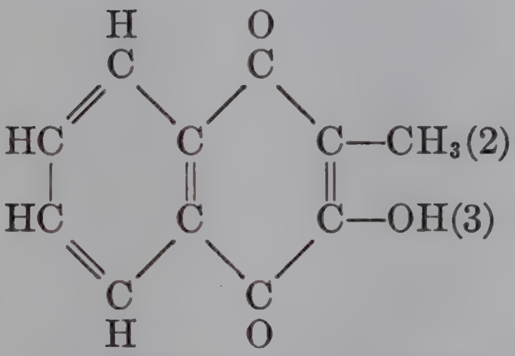
(2) Maximum Activity



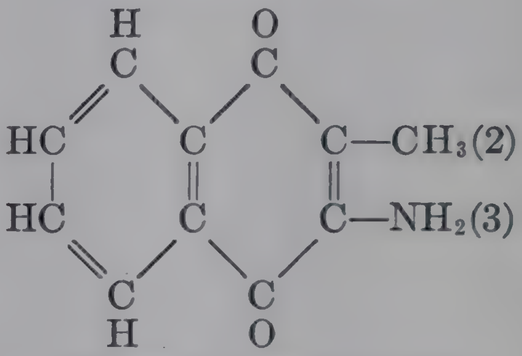
(3) Little activity



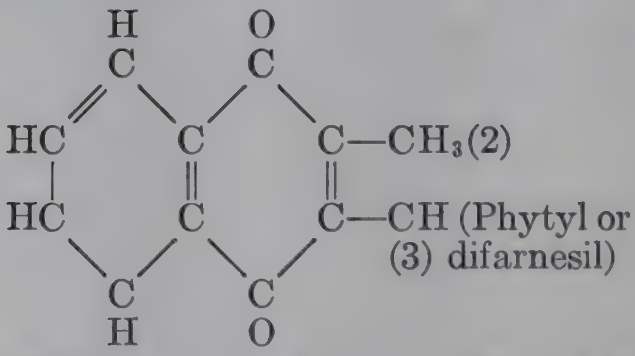
(4) Slight Activity



(5) 1/500th activity of (2)



(6) Just as active as (2)



(7) Active natural vitamins K_1 and K_2

FIG. 9. STRUCTURAL CHANGES THAT AFFECT ACTIVITY OF VITAMIN K

N. B. Note that a CH_3 group in position 2 of the nucleus appears essential to activity; that changes in the group attached to position 3 can also affect activity

manufacturers in tablets, capsules and in oil solutions. One to two milligrams is the recommended daily dose for human subjects and it is stated

(13) that the dose should not exceed 2 mg. a day and should not be continued at 2 mg. daily for a period exceeding 4 weeks. Also in cases of bile flow obstruction, bile salts should be given with it when administered orally.

Molitor and Robinson (14) using mice as the test animal found no toxic effect of vitamin K_1 even in large doses. For menadione the lethal dose was 0.5 grams per kilo of body weight; for phthiocol, 0.2 grams.

What Determines Activity?

As shown in figure 9 a methyl (CH_3) group in position 2 of the naphthoquinone nucleus appears essential to vitamin K activity. But substitutions in position 3 definitely affect activity when such substituted compounds are contrasted with menadione.

Expression of Vitamin K activity

Before the discovery of menadione it was customary to express vitamin K activity in terms of curative units. Ansbacher (15) has reviewed the various bioassay methods that have been developed since Schoenheyder (16) first outlined a test in 1936. The bioassay method measured the effect on blood clotting time (prothrombin time) and chicks were used as the test animals. The test has been modified by various investigators (17, 18, 19, 20). With the adoption of one microgram of menadione as a reference standard the amount of vitamin K substance equivalent in effect on clotting time to 1 microgram of menadione constitutes a vitamin K unit.

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Section III: Evaluation of Vitamin K for Human and Animal Nutrition

The most striking benefit of vitamin K in human physiology comes from its use in prevention of infantile hemorrhage (Hemorrhagica neonatorum) and consequent reduction in infant mortality. In the first few days of an infant's life there exists a prothrombin deficiency in his circulating blood. This hypoprothrombinemia is still more pronounced in premature infants. In their discussion of the clinical aspects of vitamin K Smith and Warner (1) say:

> "Most workers report that even in normal infants the prothrombin falls to dangerously low levels on the second, third and fourth day of life. Bleeding occurs at this time if the prothrombin level is extremely low, especially if trauma is present to provide a precipitating factor. Such trauma may be of recent origin, but in some cases the blood apparently begins to ooze again from lesions sustained during delivery. Such delayed bleeding may occur in any portion of the body but is probably more common within the cranial cavities or from tears in the liver or other abdominal viscera.
>
> Far more common than "delayed bleeding" is the "early type" suffered by the infant at birth or immediately thereafter. In this type of bleeding birth trauma is obviously a factor of great importance, but it has been suggested that the infant may already suffer from serious prothrombin deficiency at this time. Prothrombin assays made at birth support this contention."

In 1939 Waddell and Guerry et al. (2) showed that hemorrhagica neonatorum can be cured by administering vitamin K to the infant and that the vitamin prevents the fall in prothrombin level. Hellman et al. (3) demonstrated that the infant could be given protection if the vitamin was administered to the mother during the last days of pregnancy.

At the 11th Annual Meeting of the American Academy of Pediatrics (Oct. 9–11, 1941) it was agreed that the prophylactic use of vitamin K to control and prevent the hypoprothrombinemia of the newborn should be universally adopted; that every infant should be protected before birth through vitamin K administration to the mother in sufficient amounts and in sufficient time before delivery to be effective and in addition, to provide every possible protection, the infant at birth should also receive vitamin K whether or not there had been previous maternal administration.

Incidentally a daily dose of 1–2 mg. of menadione or its equivalent to the mother before birth of the infant and 0.5 to 1 mg. after birth appears customary but equivalence is important in estimating amounts of the vitamin K form used. The water soluble form for example has a higher molecular weight than menadione itself and the wt. dose is necessarily larger.

Prothrombin Time

Since the function of vitamin K is to restore blood clotting time to normal it is essential to the diagnosis of vitamin K deficiency that the prothrombin content of the blood be assayed and its clotting time determined. Quick (4a, b) has developed the test now in general use which is as follows:

a) "A drop of blood obtained by heel or ear puncture is put on a glass slide and mixed with a drop of equal size of thromboplastin. The mixture is slowly stirred with a fine pointed stirring rod. By holding the slide over a light the exact clotting time can readily be determined. Normal blood will clot in 15–20 seconds.

b) To make the thromboplastin solution: "Mix 0.3 gm. of dehydrated rabbit brain with 5 cc of physiologic solution of sodium chloride containing 0.1 cc of sodium oxalate. Incubate at 45°C. for ten minutes, then centrifuge at slow speed for three minutes to obtain a milky supernatant liquid free from coarse particles."

Allowable Therapeutic Claims for Vitamin K

Infantile hemorrhage is not the only human condition benefitted by administration of vitamin K. The Council on Pharmacy and Chemistry of the American Medical Association (5) recognizes the following claims:

"Vitamin K, both in its crude form and in certain related naphthoquinones with analagous antihemorrhagic activity seems to have a specific effect on prothrombin deficiency occurring under certain sets of circumstances:

1. In primary dietary deficiency of vitamin K which, while admittedly rare, does exist.
2. In obstructive jaundice, in which vitamin K has proved to have an extraordinary protective effect against hemorrhagic diathesis.
3. The hemorrhagic state associated with primary hepatic disease is controlled in part, but not entirely, by vitamin K and by the naphthoquinones with analagous activity. The difficulty seems to lie in the fact that the liver cannot utilize the material in the formation of prothrombin except to a limited degree.
4. The hemorrhagic states which exist in connection with certain intestinal diseases such as ulcerative colitis, sprue, and celiac disease, characterized by either a loss of continuity of the intestinal tract or by a disturbance of its absorptive surface are also affected in a specific manner by vitamin K.
5. In the treatment of the physiological hypoprothrombinemia of the newborn which exists during the first week of life, the vitamin and its analogues seem to be specific. It seems now fairly well established that the vitamin itself or the naphthoquinones, when administered parenterally to a woman during labor, in amounts as small as 0.5 to 2 mg., insures that the newborn infant will have a normal amount of

prothrombin in the circulating blood. These doses can be given parenterally to the newborn infant and produce the same effect."

Effect of Sulfa Drugs on Vitamin K

Human beings normally get their supply of vitamin K either from the diet or by synthesis in their own intestines by the intestinal bacteria. In 1936 Almquist and Stokstad (6) demonstrated that chicks fed a ration devoid of vitamin K nevertheless excreted fecal matter containing some vitamin K. They came to the conclusion that in the chick the vitamin K was actually synthesized but too far down in the gut to permit absorption into the blood. Early in 1937 Dam et al. (7) showed that rats, guinea pigs, and dogs, in contrast to chicks, could be maintained for a long time on K-free diets. By 1940 it had been demonstrated that a number of animals including man were more or less independent of vitamin K in their food since it was synthesized by their intestinal bacteria, was readily absorbable into the blood in the presence of normal bile flow, and the coliform bacteria were particularly efficient in synthesis of the vitamin.

To test this viewpoint further, experiments were conducted with certain bacteriostatic sulfa drugs. It was found that only 0.5 per cent sulfaguanidine or succinyl sulfathiazole had this effect of reducing the intestinal synthesis by rats and creating a vitamin K deficiency. But it was also discovered that the effect of the drugs could be counteracted by giving the vitamin known as paba (para-aminobenzoic acid). This effect of paba raised the question whether the effect of the drugs and of paba was confined to the gut and action of the intestinal bacteria.

The experiments of Kornberg et al. (8) and of White (9) appear to have established that the action of both the drugs and of paba is located in the gut. The action of various sulfa drugs was found proportional to their antiseptic properties as determined by White (9), and even when given subcutaneously not only was the blood prothrombin content decreased or counteracted by paba but the subcutaneous doses maintained levels of free sulfadiazine in the blood and in the cecal content similar to that produced by a diet containing 0.5 per cent sulfadiazine. When the effect was counteracted by subcutaneous administration of paba there was definite increase of paba in the cecum.

Under What conditions does the human diet need vitamin K Supplement?

Natural vitamin K_1 is present in many foods, especially leafy green vegetables. Add to this the ability of normal human beings to synthesize their needs in their own digestive tract and it is obvious that vitamin K deficiency in normal human beings will be of rare occurrence. Supplementing vitamin K therapy then becomes necessary only when these sources of vitamin K fail or there is failure to absorb and utilize the supply. In general such conditions are as follows:

1. The diet lacks vitamin K or the material from which the bacteria can make it.
2. There is failure of bile supply to insure proper absorption of the vitamin from the gut such as occurs in bile flow obstruction or liver diseases of a certain type.
3. When there is damage to the gut wall that prevents passage of the vitamin through it such as occurs in colitis or other conditions in which the mucosa is damaged.
4. When liver damage is such that it cannot form prothrombin even though supplied with adequate amounts of vitamin K.
5. It has also been shown that retinal neonatorum hemorrhages are preventable by administration of vitamin K to mothers (10).

In the normal human being the prothrombin content of the blood is always in excess of the amount necessary to normal coaguability of the blood. Furthermore it is possible to have a 20 per cent drop in normal content before clotting time is appreciably prolonged and serious hemorrhage will not occur until this low level is reached.

Vitamin Requirements Of Domestic Animals

As has already been explained, a hemorrhagic disease in chicks led to the discovery of vitamin K and its function. In chicks, then, a dietary lack of vitamin K is serious; the chicks may bleed to death from any injury which causes rupture of the blood vessels. Mature birds, however, do not appear to be as sensitive to lack of vitamin K in the diet, perhaps due to better absorption of their intestinally produced supply (6, 7).

But, it has been shown that laying hens fed a vitamin K deficient diet produce eggs low in K content and that when these eggs are incubated the chicks hatched from them have low reserves of the vitamin and may bleed to death from as simple a trauma as wing banding.

As would be expected from the nature of their diet, and because of rumen synthesis of vitamin K, herbivorous animals such as cattle, sheep and swine are rarely subject to vitamin K deficiency disease.

Food Sources of Vitamin K

Since mammals appear to have a sufficient supply of vitamin K provided by bacterial flora and since synthetic vitamin K can be prepared cheaply there has been little incentive to obtain information as to its distribution in foods and data are scanty. Fruits and cereals are poor sources, green leafy tissues are the richest natural sources.

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CHAPTER VI. THE WATER-SOLUBLE VITAMINS

In 1916 McCollum and Kennedy (1) suggested that a water-soluble growth-promoting factor such as occurred in Osborne and Mendel's (2) 'protein-free milk' and which also cured the disease called beri-beri or polyneuritis should be called *unidentified dietary factor water-soluble B*.

Funk (5) and Sherman and Smith (6) have fully covered the historical steps leading from Takaki's (3) demonstration that the beri-beri of the Japanese Navy in 1878 could be prevented by inclusion in the diet of certain foods; the location of the vitamin in rice polishings and yeast, to the climax of Funk's (4) isolation of what he called "vitamine".

By 1916 it was recognized that it was possible to extract with water from various plant and animal materials a substance that was both growth-promoting and beri-beri curative, that rice polish and yeast were particularly rich sources of the factor, but that it was also rather widely distributed in natural food stuffs. At the time it was generally assumed that such water extractions yielded a single substance, probably identical with Funk's "vitamine".

The first indication that there was more than one water-soluble vitamin came with the studies begun in 1912 by Holst and Frohlich (7) and led to the identification of a water-soluble scurvy-preventive vitamin called vitamin C. The chemical identification of this vitamin however, did not develop until 1933 (8).

The Vitamin B Complex

The suggestion that water-soluble B was not a single dietary factor came first in 1919 and 1920. In 1919 Mitchell (9) questioned the identity of the water-soluble growth-promoting vitamin and the anti-beri-beri vitamin. In 1920 Emmett and Luros (10) showed that heat treatment of yeast destroyed its anti-beri-beri action without destroying its growth-promoting effect; indicating that what had been called single vitamin B was actually a mixture of a heat-stable and a heat-labile fraction.

In 1901 Wildiers (11), working in Ide's laboratory in Louvain, Belgium, took issue with Pasteur's claim that one could grow yeasts on a synthetic medium containing simply sugars, ammonium tartrate, and yeast ash. Wildiers postulated that in addition to the ingredients named by Pasteur the wort needed minute amounts of organic substance to which he gave the name "bios".

In 1919 Williams (12) suggested that Wildiers' bios and water-soluble B were identical substances. He supported this claim by showing that the substance stimulating the growth of yeast in a sugar-mineral culture medium occurred in the same materials that yielded water-soluble B; that the 'bios' had properties similar to vitamin B in distribution, solubility, adsorption on fullers earth, heat stability and behavior toward acids and alkalies.

Eddy and Stevenson (13) reviewed Williams' claims, using a Gebrüder Mayer strain of yeast and water extracts of certain sources that had been assayed previously for water-soluble B content by animal growth tests and reported by Osborne and Mendel (14). They failed to get satisfactory correlation between bios effect and B content. As a result they attempted the isolation from yeast of a true bios. In 1934 Eddy et al. (15) reported isolation of a crystalline product with ability to promote growth of yeast in doses as small as .005 mg. per ml. of culture medium. It was later learned that the bios effect was producible by Williams' pantothenic acid in an amount as small as .0000008 mg./ml. and an even lower concentration of biotin. Williams (16) has stated that these later discoveries indicate that the Eddy, Kerr, Williams bios might have been 99.9 per cent pure and still contaminated with enough pantothenic acid to give the growth effect they got.

As a matter of record the first grantedly pure bios was isolated in W. L. Miller's laboratory by Eastcott (17) and is now known as inositol. Williams pantothenic acid and biotin were discovered later (*circa* 1940).

These searches for bios have a special significance since for the first time they introduced the use of response of micro-organisms for vitamin detection. As Elvehjem stated in a review in 1943: "Half of the B vitamins were first recognized as growth factors for bacteria."

Incidentally some concern has been expressed as to whether these micro-organism stimulants are entitled to the term 'vitamin'. As Williams puts it: "Especially in recent years the term vitamin has been used almost universally to designate organic substances which in minute amounts are of importance in *animal* nutrition. Because of this fact the term *nutrilite* was coined by myself (1928) to include organic substances which in minute amounts function in a similar manner to other types of organisms. The term 'vitamin' carries unfortunate etymological implications and its use should not be unduly extended. The term nutrilite on the other hand, is not objectionable from this standpoint, since it implies only an importance in nutrition and nothing as to the chemical nature of the agent."

Though some were identified as growth factors by micro-organism response, the water-soluble vitamins listed at the end of this chapter have all been essential to animal nutrition and are therefore apparently entitled to the vitamin classification. However, in the case of inositol and choline there may be some question. The amounts of these needed are much in excess of the stimulatory amounts necessary for usual vitamin effect. The situation in their case is somewhat similar to the removal of *vitamin F* from the list of vitamins when it was found that the effect it produced was due to presence of certain unsaturated fatty acids already well known in the literature.

Search for the Anti-Pellagra Vitamin

In addition to the search for a bios, a similar search for a vitamin preventive and curative of the disease called pellagra contributed to the splitting of water-soluble B into the present B-Complex Group.

Following the demonstration of a heat-stable and heat-labile fraction of vitamin B as stated above, Goldberger and Lillie (18) in 1926 described a condition they called rat-pellagra which they claimed to be caused by lack of the heat-stable vitamin B since it occurred in rats fully supplied with the anti-beri-beri factor. Pursuit of this line of investigation led ultimately to the identification of three new members of the complex, viz., Riboflavin or vitamin B_2, Pyridoxine or Vitamin B_6 and Niacin or vitamin P-P.

Today's Water-Soluble Vitamins

There is no reason to believe that the following list of water-soluble vitamins is complete. Liver, in addition to yeast, has been a rich source of new factors of the B Complex and there is every reason to expect further additions as research progresses. The following are those that have been definitely identified chemically and in most cases the identification confirmed by synthesis:

Vitamin B_1 or Thiamine
Vitamin B_2 or Riboflavin
Vitamin B_6 or Pyridoxine
Vitamin P-P or Niacin
Pantothenic Acid or Pantothen
Inositol
Para-amino-benzoic acid or Paba
Biotin
Choline
Folic Acid or the Pteroyl-glutamates
Vitamin C or Ascorbic Acid.

With satisfactory chemical identification there is today a tendency to drop the letter designation and to substitute a descriptive name that indicates the chemical nature of the vitamin. Also the use of terms such as anti-beri-beri, anti-pellagric etc. is too limited to cover all the functions of the respective vitamins.

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CHAPTER VII. VITAMIN B_1 (THIAMINE)

SECTION I. FUNCTIONS OF THIAMINE

Vedder (1) has reviewed the pathology of beri-beri and defines it as follows:

> "Beri-beri is a disease resulting from faulty metabolism and is directly caused by a deficiency of the anti-neuritic vitamin B_1 and possibly other deficiencies in the diet. Among Orientals, this deficiency is usually produced by the too exclusive use of decorticated or polished rice; but it may equally be caused by the too exclusive use of wheat flour and other carbohydrate staples. Clinically, beri-beri is characterized by degenerative changes in the nervous system including a multiple peripheral neuritis, which may exist alone but is often combined with generalized edema and serous effusions, and by a tendency to the development of cardiac hypertrophy, which frequently results in cardiac failure and sudden death. In this definition vitamin B_1 as distinguished from other fractions of the vitamin B complex is the compound isolated and synthesized by R. R. Williams, a compound of pyrimidine and thiazole which has been designated by Williams as thiamine."

A prime function of thiamine which led to its isolation and final synthesis, then, is its ability to prevent and cure beri-beri.

The function that led to its designation as water-soluble vitamin B was its growth stimulating activity and one of the earliest manifestations of vitamin B_1 deficiency observed was loss of appetite or anorexia. In 1917 Eddy and Roper (2) reported successful use of the vitamin extracted from sheep pancreas to counteract loss of appetite in infants suffering marasmus, and in the same year Osborne and Mendel (3) reported that food consumption of rats was directly proportional to the amount of vitamin B in the diet. Daniels et al (4) confirmed the effect on infants and Karr (5) in 1920 reported that the urge of a dog to eat bore a direct relation to its intake of vitamin B.

Relation of Thiamine to Calorie Intake

In 1914 Funk (6), by contrasting the effect of diets with a high percentage of carbohydrate with those high in fat or protein, showed that the onset of beri-beri came much earlier on the high carbohydrate diet. This was the earliest suggestion that thiamine needs were related to the character of food fuels used and to carbohydrate fuel in particular.

In 1934 Cowgill (7) published a summary of a series of important studies that definitely related thiamine requirement to calorie intake. He derived a mathematical expression for animal need of thiamine based on animal weight and calorie intake. At the time pure thiamine was not available and Cowgill did his experimenting with a vitamin B_1 concentrate which later proved to contain 0.15 micrograms of thiamine per milligram. Cowgill' original formula was:

Vitamin B_1 requirement per day in milligrams of concentrate
$= K_s \times$ Wt. in grams $\times$ calorie intak

In this formula K_s was a constant for a particular species of animal e.g.

K_s for the mouse was 0.15

K_s for the rat was 0.0099
K_s for the pigeon was 0.0037
K_s for the dog was 0.000076
K_s for man was 0.0000284.

When the thiamine content of Cowgill's vitamin B_1 concentrate wa established it became possible to rewrite his formula as follows:

Human daily need of thiamine in micrograms
$= .00213 \times$ wt. in pounds $\times$ calorie intake

For a 150 lb man eating 2400 calories of food a day this totals 767 mcg The Food and Nutrition Board of the National Research Council today (1948) recommends as a daily allowance for such an individual 1200 micrograms, but this allowance carries a factor of safety. In Section III the present evidence on human thiamine requirement is discussed, but Cowgill's work first firmly established that the need was controlled by the calorie intake.

But, with this need established explanation of how thiamine functions in the metabolism of fuel foods was still lacking.

The Role of Thiamine in the Metabolism of Carbohydrate

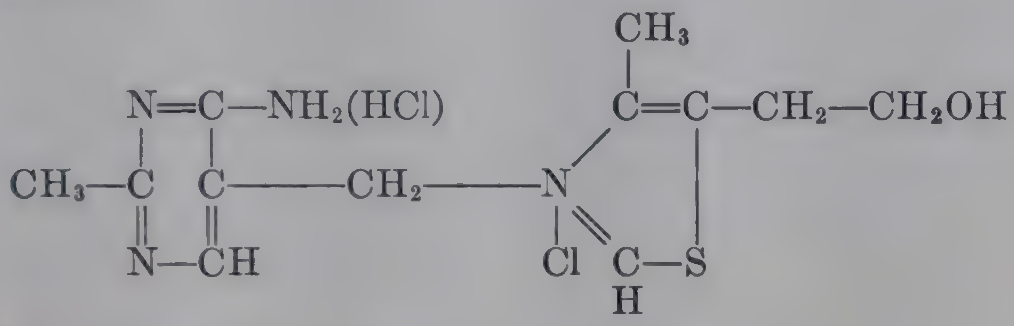
In 1930 Kinnersley and Peters (8) noted a spasm produced in pigeons by an overdose of insulin and this observation led them to initiate an investigation of the relation of thiamine to brain carbohydrate metabolism in normal and in vitamin B_1-deficient pigeons. It was well known that normal brain tissue *in vitro* passed through stages of glycolysis with the breakdown of glucose to lactic acid. When the brain tissue of B_1-deficient pigeons was subjected to the same procedure, it was noted that there was an abnormal increase in lactic acid; also that the addition of vitamin B restored tissue respiration in the presence of lactic acid but not unless lactic acid was present. This led to search by Peters et al (9) for another intermediate which they identified as pyruvic acid.

Correlated with these observations of Peters et al came the demonstration by Platt and Lu (10) that beri-beri cases showed an increase of pyruvic acid in the circulating blood; increase in blood pyruvate is today used as diagnostic sign of beri-beri.

In the metabolism of glucose by yeast it is known that a final step in the process is the formation of pyruvic acid and its conversion into water and carbon dioxide by decarboxylation and oxidation; that this reaction is produced by respiratory enzymes in the yeast.

In 1911 Neuberg and Karczag (11) reported the presence in yeast of a carboxylase enzyme. This carboxylase enzyme was obtained in highly purified form by Green et al (12) and by Kubowitz and Luttgens (13) in 1941. Before its composition was worked out Auhagen (14) as early as 1933 had shown that the carboxylase required a thermo-stable coenzyme to act, and in 1937 Löhman and Schuster (15) isolated this coenzyme from yeast as a crystalline hydrochloride. Comparison of this coenzyme with Williams' thiamine showed it to be phosphorylated thiamine. (See fig. 10.)

A. Thiamine HCl



B. Co-Carboxylase (Diphospho-Thiamine)

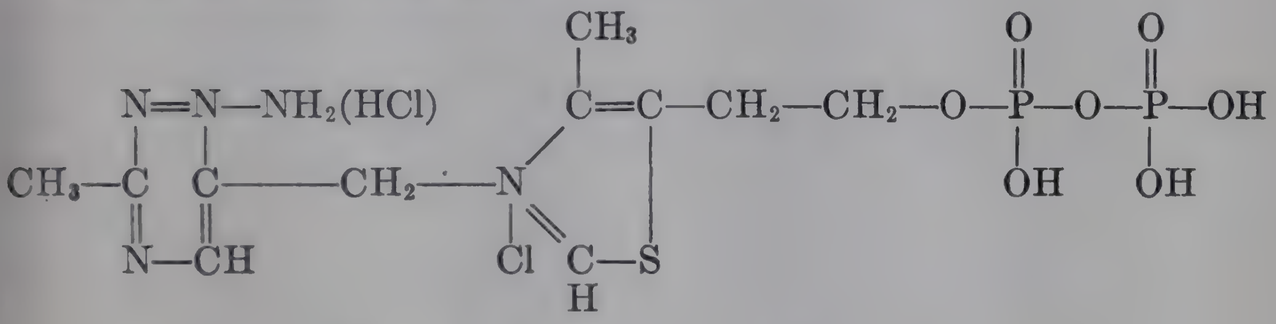


Fig. 10. Co-Carboxylase & Thiamine

The carboxylase enzyme itself appeared to be a protein-diphosphothiamine-magnesium complex.

These accumulated studies appear to clarify the role of thiamine in carbohydrate metabolism. They indicate that thiamine functions (after phosphorylation) as the prosthetic group of a respiratory enzyme. By its action, when glucose is present, the glucose is converted through the pyruvate stage to CO_2 and water with liberation of energy. When thiamine is lacking or in inadequate amount there is accumulation of pyruvic acid and failure to obtain the full energy value of the ingested carbohydrate.

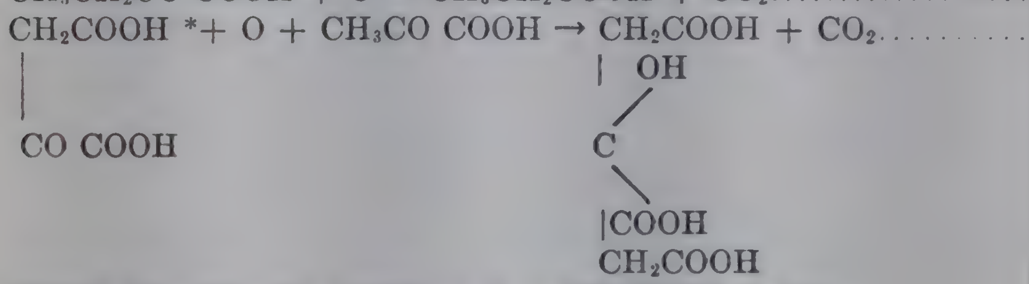
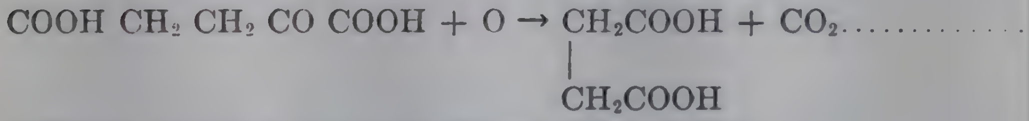
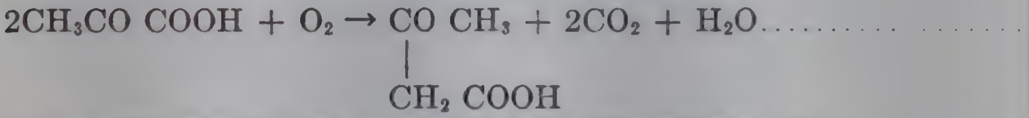
The exact path of conversion of pyruvic acid in human tissues has not been fully clarified but that the thiamine is first phosphorylated and then acts as a coenzyme is apparently completely established today.

According to Lipton and Elvehjem (16) and Weil-Malherbe (17), the transfer of labile phosphate groups from the nucleotide adenosine triphosphate accomplishes the phosphorylation of thiamine in yeast. It is suggested that ingested thiamine is first changed in the animal body to the phosphorylated form (co-carboxylase) which then becomes a part (prosthetic group) of the holo-enzyme carboxylase. When thiamine is fed and absorbed from the digestive tract it is largely converted to the co-carboxylase form and stored as such in the liver and kidney. As it is needed in the tissues it is dephosphorylated and travels in the blood as free thiamine.

TABLE 25

Some reactions in which diphosphothiamine participates

(After Ochoa (18) 1942)

Reactions	References
1. $CH_3CH_2CO\ COOH + O \rightarrow CH_3CH_2COOH + CO_2$	(19)
2.  $\begin{array}{l}CH_2COOH\\ \vert\\ CO\ COOH\end{array}\ {}^{*}+ O + CH_3CO\ COOH \rightarrow \begin{array}{l}CH_2COOH\\ \vert\quad OH\\ \ \ \diagup\\ C\\ \ \ \diagdown\\ \vert COOH\\ CH_2COOH\end{array} + CO_2$	(20) (21)
3.  $COOH\ CH_2\ CH_2\ CO\ COOH + O \rightarrow \begin{array}{l}CH_2COOH\\ \vert\\ CH_2COOH\end{array} + CO_2$	(22) (23)
4.  $2CH_3CO\ COOH + O_2 \rightarrow \begin{array}{l}CO\ CH_3\\ \vert\\ CH_2\ COOH\end{array} + 2CO_2 + H_2O$	(21)
5. $CO_2 + CH_3CO\ COOH \rightarrow COOH\ CH_2\ CH_2\ COOH$ (?)	(24) (25)
6. $CH_3COOH + 2O_2 \rightarrow 2\ CO_2 + 2\ H_2O$	(26)

In the tissues it is probably again phosphorylated. When excreted in the urine, it is again in the free thiamine form.

Ochoa (18) has summarized these findings as follows:

> "We have seen that diphospho-thiamine (co-carboxylase) is essential for the decarboxylation of pyruvic acid in yeast and for its oxidation in animal tissues and in certain bacteria. Through the agency of diphosphothiamine is probably evolved all the carbon dioxide of alcoholic fermentation and a large part of the carbon dioxide of respiration. Decarboxylation of other alpha-keto acids also requires diphosphothiamine. It would seem that diphosphothiamine is primarily concerned with the decarboxylation of alpha-keto acids in all living cells." (See Table 25.)

At one time it was suggested that diphoshphothiamine might act as a hydrogen carrier in biological systems through alternate oxidation and reduction of the quaternary thiazole nitrogen but this viewpoint has been abandoned (18).

Relation of Thiamine to Types of Calorie Foods

There is a definite evidence that fats and alcohol in isocaloric portion with carbohydrate require less thiamine for their metabolism than carbohydrate. Therefore considerable argument has arisen whether in calculating the thiamine need the requirement should take into consideration total calorie intake or only carbohydrate calorie intake. There has also been questioned whether weight is important; Cowgill made it a factor in his thiamine requirement formula. The consensus of opinion at present appears to be that in determining thiamine need it is justifiable to base it on total calorie intake. In planning the suggested allowances set up by the Nutrition Board of the National Research Council the Board took the view that the daily allowance be calculated on the basis of 0.5 milligrams per day for each thousand total calorie intake up to 3000 calories; 0.3 milligrams per thousand calories for intakes over 3000 calories; and on this basis regardless of age, sex or size. (For further discussion of how human needs have been estimated see Section III.)

Effect of Other Components of the Diet on Thiamine Requirement

The sparing action of fat in the diet on thiamine requirement has been reported by several investigators (27, 28, 29, 30). Various explanations have been offered. Westenbrink (31) suggests that when fat is fed the organism uses in its metabolism less thiamine than it requires for carbohydrate metabolism. Whipple and Church (32) believe thiamine necessary for fat synthesis, but that in thiamine deficiency the rat preferably metabolizes fat as opposed to carbohydrate. Barelare et al (33) have shown that, given free choice, rats on a thiamine deficient diet show an increased appetite for fat.

At one time it was held that consumption of alcohol increased thiamine requirement but later evidence (34, 35), indicates that it actually decreases the requirement. It would therefore appear that, like fat, alcohol actually requires less thiamine for its metabolism than carbohydrate. However one effect of alcoholism is to reduce food intake and hence reduction of B_1 intake. As B_1 stimulates appetite it helps restore the alcoholic to normal food intake.

Thiamine and the Gastro-Intestinal System

It has already been noted that one of the earlest manifestations of thiamine deficiency to be observed was anorexia, or loss of appetite, and its response to thiamine therapy. In 1916 Carlson (36) showed that the sensation of hunger was producible by contractions of the walls of the empty stomach. It was therefore logical to investigate the effect of thiamine on gastric motility. Cowgill et al in 1926 (37, 38), demonstrated production of gastric atony and hypomotility and also reduction in gastric secretion in vitamin B deficiency. Sparks and Collins (39) have given further evidence of a specific effect of thiamine on the intestinal musculature in thiamine deficiency.

McCarrison (40) was the first to report gastric erosions and ulcers in vitamin B deficiency. Thatcher et al (41) reporting on a study of avitaminoses in 1938 stated:

> "Perhaps the most significant result in the investigation is the finding of gastric ulcers as the result of the specific influence of a deficiency of vitamin B_1."

Drummond (42) gave further evidence based on a study of over 1000 rats of the production of gastric ulcers by thiamine deficiency, but Dalldorf (43) who concurs with the results in rats states:

> "We have found no record of gastric ulcers in human cases in thiamine deficiency. The mechanism by which they occur in rats is obscure. . . . Considerable evidence exists to show that the terminal nerve structures in the gastric wall are diseased in peptic ulcer. Stohr's studies have confirmed this and shown that these minute lesions are widely distributed in the gastric wall. The cells of both Auerbach's and Meissner's plexi are altered, an observation made in experimental thiamine deficiency by McCarrison."

Thiamine Deficiency and Anatomical Lesions

The known anatomical lesions resulting from thiamine deficiency are polyneuropathy, the ophthalmoplegia of Wernicke's syndrome, and circulatory disturbances. Jolliffe (44) states:

> "The neurological manifestations of vitamin B1 deficiency whether in a mild or severe form, are bilateral and symmetrical and characteristically involve first and predominantly the lower extremities. Peripheral neuritis that involves a single nerve, or that is not bilateral and symmetrical, or that does not involve first and predominantly the lower extermities, is not, in my experience, due to vitamin B_1 deficiency."

The Council on Pharmacy and Chemistry of the American Medical Association (45) limits its acceptance of thiamine usage for treatment of neuritic manifestations as follows:

> "While it has not been established that thiamine deficiency is the sole cause of conditions described as alcoholic neuritis, the neuritis of pregnancy, and the neuritis of pellagra, there is some definite evidence of value of this vitamin in the treatment of these conditions. *Vague representations with respect to the value of thiamine in the treatment of other types of neuritis are not permissible.*"

Thiamine and Neuropathy

There is convincing evidence today that thiamine deficiency affects the finer structure of nerves and produces nerve lesions (46). How this effect is produced has been the subject of much research and controversy. Dalldorf (43) in 1944 commented on the situation as follows:

> "Whether the lesions are due to inadequate carbohydrate oxidation or to the accumulation of toxic metabolites is not known .... deficiency operating through a toxin would explain many features of the disease."

The toxin theory has been studied extensively but without conclusive evidence to support it.

Engel and Philips (47) have even gone so far as to question the right of the vitamin to the term "antineuritic" and to suggest that the observed nerve lesions are due to lack of other dietary factors than thiamine, but this viewpoint seems untenable (46) though there is no question that other vitamins are concerned in food fuel metabolism and in disease manifestations.

One of the more recent attempts at explanation has been advanced by von Muralt (48). His viewpoint is that thiamine is an actual metabolite, not simply a catalyst, involved in nerve metabolism. As he puts it:

> "Because it is of such importance, the nervous tissue is one of the last tissues to lose its thiamine content when a state of avitaminosis prevails. When it is losing its thiamine, the general disarrangement of the body is so far advanced that it is hard to distinguish between specific and general effects. . . . Why thiamine is liberated in the normal excitation process and why it is accumulated in monoiodoacetic acid poisoned nerves and disappears on excitation, is a problem which needs further experimental work. It may be related to the reaction:
>
> Adenosine triphosphate + thiamine = adenylic acid + cocarboxylase. It has been suggested that this mechanism is essential for the formation of acetyl-choline (49). The scheme which was visualized is the following: The breakdown of adenosine triphosphate activates thiamine to cocarboxylase which catalyzes the anaerobic and aerobic decarboxylation of pyruvic acid in two ways:
>
> 1) Anaerobic
>
> $2\ CH_3CO\ COOH + H_2O \rightarrow CH_3COOH + CH_3CHOH\ COOH + CO_2$
>
> 2) Aerobic
>
> $2\ CH_3CO\ COOH + O_2 \rightarrow 2\ CH_3COOH + CO_2$
>
> These reactions furnish the acetic acid necessary for the acetylation of choline derived from the dephosphorylation of nerve phosphatides. Adensosine triphosphate and cocarboxylase act as phosphate donor and acceptor, and are connected with the degradation of glucose, acting as energy transmitters. . . . Thus far thiamine and cocarboxylase have been considered as catalysts. This view does not correspond to the rather large daily requirement of thiamine in the body. Thiamine must also be considered as a metabolic substance."

Further study of the action of thiamine in nerve metabolism may clarify its relation to particular forms of neuritis. At present the polyneuritis of beri-beri and pellagra are accepted as consequences of thiamine deficiency. Wernicke's syndrome, characterized by clouding of consciousness, ataxia, and paralysis of the ocular muscles (usually but not always associated with alcoholism), has responded to thiamine therapy and there is consistent evidence that this syndrome is associated with disturbance of pyruvic acid metabolism.

Alcoholic neuritis was originally attributed to the toxic action of alcohol. When crystalline thiamine became avaiable Goodhart and Jolliffe (50) tested its effectiveness in treatment of mild cases of alcoholic polyneuroppathy. They found that when 10 mg of thiamine was administered daily to subjects receiving high vitamin diets, both rate and degree of recovery were significantly increased over those of a control group which received only the high vitamin diet. Other studies (51, 52), have strengthened the view that thiamine deficiency has a direct causal relation to alcoholic neuropathology.

Like alcoholic polyneuritis the neuritis of pregnancy, usually associated with excessive vomiting, was also originally considered to be caused by a toxin. That thiamine deficiency may be at least one factor causative of this polyneuritis is now generally accepted but it is conceded that it may not be the sole factor involved.

Diabetic neuritis, on the other hand, is today considered due to other causes than thiamine deficiency. Gordon and Sevringhaus (53) comment on this as follows:

> "Mention should be made of the role of thiamine in the treatment of diabetic neuritis. The frequent failure of this condition to respond to added supplements of this substance is well known and may probably be attributed to the association of the nerve lesion with a deficiency of other members of the B complex than thiamine. In many cases there may be no deficiency; in these, therapy of this type could hardly be expected to produce satisfactory results."

Needles (54) also reports failure of thiamine therapy in his cases of diabetic neuritis.

Thiamine and Neurasthenia

Quite apart from specific neuritis one of the recognized effects of thiamine deficiency is the production of certain physical states that are generally classed as neurasthenia or as Jolliffe (44) puts it:

> "Disturbances in function due to thiamine deficiency are probably and logically more common than the recognized anatomic lesions. Since these symptoms are found only in patients 'in whom nothing organic can be found' they have heretofore been included in the diagnostic grab-bag of 'neurasthenia'."

The syndrome consist of tension and irritable weakness. It is manifested in complaints of easy tiring, weakness, exhaustability, head pressures, poor sleep, feeling of tenseness and irritability, various aches and pains, subjectively poor memory and difficulty in concentration. Jolliffe and coworkers (55) were able to produce this syndrome in human subjects by a thiamine deficient diet and to remove all the symptoms in a short period with thiamine therapy. Williams, Mason, Wilder and Smith (56) reported similar experiments with good response to thiamine therapy and Wilder suggested the term "morale vitamin" for thiamine.

It should not be inferred, however, that all neurasthenia is traceable to thiamine deficiency. Thiamine therapy is valid only when there is proof that there is a thiamine deficiency and the patient responds to its administration.

Thiamine and Circulatory Disturbances

In acute beri-beri disease death is usually due to heart failure. A definite relation of thiamine deficiency to cardiac function has long been recognized. Drury et al (57) devised a thiamine assay technic based on the fact that in the rat thiamine deficiency produces an abnormal infrequency of the heart beat (bradycardia). The characteristics of beri-beri heart in human subjects are enlargement (usually of the right side), decreased circulation time, elevated or normal venous pressure, less difference than normal between the oxygen content of arterial and venous blood, and usually rapid recovery following thiamine therapy. Similar effects are observed in patients developing thiamine deficiency through the excessive use of alcohol. In both these cases the heart is tremendously dilated and the edema is massive.

Weiss and Wilkins (58) were among the earliest to study the causes of these cardiac disturbances, and Porter and Downs (59) confirmed their findings. They agreed that the most satisfactory explanation for the altered circulatory dynamics in patients with beri-beri heart is that there is failure of the myocardium, an increase in volume of the peripheral vascular bed caused primarily by dilatation of the arterioles.

The effect of thiamine was interpreted as increasing arterial tone and thus decreasing the volume of the peripheral bed so that the amount of blood returning to the heart per minute was lowered and the work of the myocardium reduced. It appeared that there was also improvement of myocardial function by direct action of thiamine on the heart.

Swank and Bessey (60) further confirmed these observations by producing similar cardiac changes in pigeons through thiamine deficiency and their cure by thiamine therapy. In discussing the mechanism involved, they suggest that tachycardia is due to vasodilation which in turn is caused by accumulation of the intermediate products of carbohydrate metabolism.

At present however it is still impossible to be certain of the exact role that thiamine plays in causation of cardiac conditions and the relative role of other deficiencies and of factors other than nutritional.

Thiamine and Glandular Secretions

Adrenal hypertrophy (61) and suppression of follicular function (62) and effect on lactation (63, 64), have all been reported as manifestation of vitamin B_1 deficiency. Unfortunately the experiments reporting these effects were in most cases conducted before pure thiamine was available and need further confirmation. There appears to be definite relation between the activity of the thyroid gland and vitamin B_1 requirement. Himwich et al (65) report that dogs fed thryoid gland require an increased amount of vitamin B_1. Similar findings have been reported with pigeons (66) and others (67, 68), also support the claim that thyroid activity affects vitamin B_1 requirement. Blaizot-Guenot (71) has reported that male rats given thyroxine or deprived of vitamin B_1 develop creatinuria. The creatinuria produced by the thyroxine was antagonized by giving thiamine (200 mcg thiamine neutralized the effect of 10 mcg of thyroxine). Their explanation is that avitaminosis B_1 produces the creatinuria by stimulating the thyroid and creatinuria did not develop in vitamin B_1 deprived rats previously thyroidectomized.

McHenry (69) has shown that the oral administration of thiamine greatly increases the deposition of fat in the liver if the diet is low in choline. It has also been reported that thiamine deficiency observed in the monkey, fowls, and pigeons produces atrophy of the testes in males and affects estrus in the females.

Thiamine and Intelligence

Harrell (70) has presented evidence based on the use of intelligence tests, reaction time, and reading and arithmetical skill tests that appears to indicate that as low a dosage as 2 mg of thiamine a day can improve mental ability. Statistically such administration was followed by improvement in the treated cases. In these cases the effects may be traceable to better utilization of food and lessening of fatigue. Robertson et al (72) have reported administration of thiamine to one half of 36 pairs of identical twins (2 mg daily for four and a half months). They got with the treated individuals improvement in height and weight, in normal dexterity, and in prolonged memory tests. The treatment had no effect on vision, intelligence, reasoning, arithmetic ability, rote memory or code substitution scores.

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Section II. Forms and Chemistry of Thiamine

The isolation, chemical identification and synthesis of thiamine by R. R. Williams and coworkers (1) was the culmination of a long series of attempts to obtain the anti-neuritic vitamin B_1 in pure form. Of historical interest in this series should be mentioned the following:

1. Funk's (2) crystalline antineuritic crystals obtained in 1911 from rice polish the amino content of which led to his calling it vitamine; also his isolation of similar crystalline material from yeast in 1912. Further study of Funk's crystalline material, though it was undoubtedly curative of beri-beri, showed it was not the pure vitamin.
2. Suzuki, Shimmamura and Ohdake's (3) isolation of what they called oryzanin from rice polish in 1912. Ten mg of their product protected pigeons from experimentally induced beri-beri. It obviously contained the vitamin in high concentration but it was not the pure vitamin.
3. Seidell's contribution to the methodology of isolation (4) by adsorption on fuller's earth. Later Williams developed another aid by use of quinine to elute the vitamin from the adsorbent mixture.
4. The long series of studies in the Dutch and British East Indies and Jansen and Donath's (5) first successful isolation of the pure vitamin.

The first successful isolation of the vitamin in pure form was by Jansen and Donath, a yield of a few milligrams from a large amount of rice polish. In fact the yield was so small as to make analysis difficult and in their analysis they missed the presence of sulfur in the compound. Application of their method to yeast in Windaus' (6) laboratory in Göttingen a few years later yielded similar crystalline vitamin but Windaus found that in addition to carbon, nitrogen, hydrogen and oxygen the compound contained sulfur. They suggested the empirical formula $C_{12}H_{17}N_2O_2$.

Williams' Isolation of Pure Thiamine

R. R. Williams has described (1) in detail his procedures and they need not be repeated here. Between the years 1930 and 1934 Williams and coworkers developed a method of securing consistent yields of the crystalline vitamin from rice polish of approximately 5 grams per ton; about 25 per cent of the total content. An important step in the chemical identification was achieved by splitting the compound without loss into its two chief components; pyrimidine and thiazole. The reaction took place as follows:

$$C_{12}H_{18}N_4OSCl_2 + Na_2SO_3 \rightarrow C_6H_9N_3SO_3 + C_6H_9NSO + 2NaCl$$

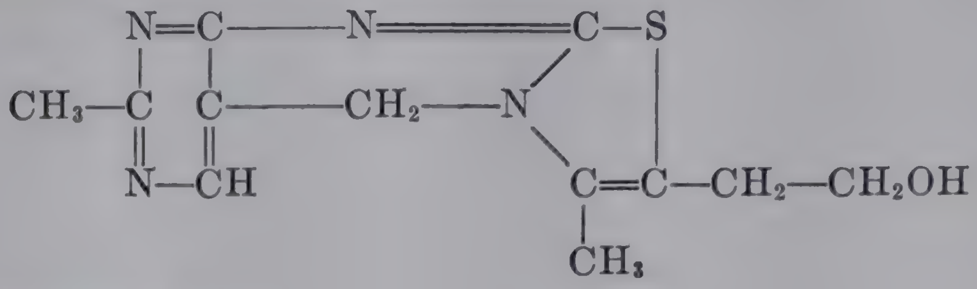
From study of these cleavage products Williams and coworkers reached the structural composition shown in figure 11. Williams' formula was later confirmed by laboratory synthesis (7).

Forms of Thiamine

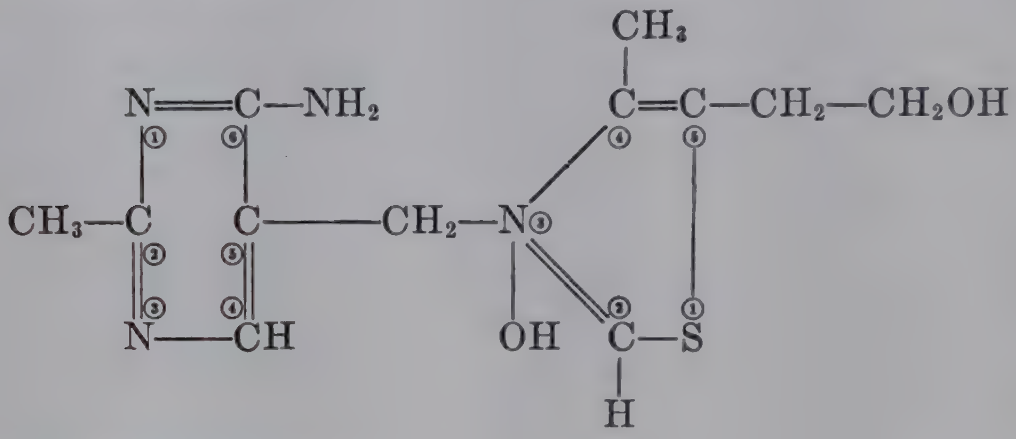
As already explained, thiamine exists in the body in two forms: the free base and the phosphorylated co-carboxylase.

In 1935 Kuhn et al (8) isolated from yeast a yellow, basic substance whose neutral or alkaline solutions were characterized by an intense blue fluorescence. They called this substance 'Thiochrome'. Barger, Bergel and Todd (9) showed that this substance could be formed from thiamine by the oxidation of an alkaline solution of the vitamin with potassium ferricyanide. This discovery made possible a photometric assay method for determining thiamine content. The method in use in this country is that worked out by Cerecedo and Hennessy (10).

A. Thiochrome



B. Free Thiamine Base



C. Pyri-thiamine or Pyramin

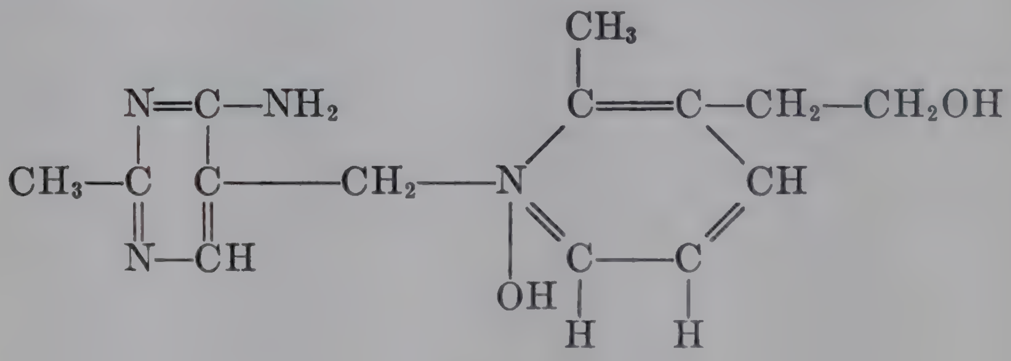


Fig. 11

Properties of Thiamine

Thiamine hydrochloride, the form in which the synthetic product is merchandised, is a white crystalline powder having a faint yeast-like odor and a salty taste. One gram of the hydrochloride is soluble in 1 cc. of water;

in about 100 cc. of 95 per cent alcohol and about 20 cc. of glycerine. It is practically insoluble in benzene and ether.

The aqueous solution of thiamine hydrochloride is acid (pH 3.5 at a concentration of 1:20). At this pH the aqueous solution can be sterilized at 120° C for twenty minutes without loss of activity. But if the solution is above pH 5.5 there will be activity loss on heating. The dry crystals are quite stable to heat; 24 hours at 100° C without activity loss. The product is not senitive to atmospheric oxidation. The free base has the structure shown in figure 11.

The hydrochloride has two absorption bands at 235 and 267 mμ. It crystallizes in acicular needles (the hemihydrate) with a melting point of 248–250° C.

In Europe the vitamin was named aneurin by Jansen. In this country the term proposed by Williams is preferred, as it shows the composition rather than the function of the vitamin.

Anti-Thiamine Compounds

Many enzymes possess a prosthetic group which appears to engage the substance acted upon (substrate) by absorption both of the prosthetic group and substrate on the same protein surface. For a given enzyme, specificity results because only substances with a particular structure can be adsorbed and made available for reaction. It has been discovered that there exist substances with a chemical structure sufficiently similar to the normal prosthetic group of an enzyme to allow their adsorption but sufficiently different so that the chemical reaction does not occur; substances that compete with the normal enzyme and, if adsorbed, block its action. The similarity of sulfanilamide to the vitamin paba blocks the latter's growth-stimulating action on certain bacteria. Another example is salicylic acid which blocks the action of vitamin K and produces hypoprothrombinemia.

Wooley and White (11) have demonstrated that a pyridine analogue of thiamine (pyrithiamine or pyramine) produces a thiamine deficiency in mice by such an action. Mice fed large amounts of thiamine and small amounts of pyrithiamine do not show the deficiency, but if the ratio is raised to 20–30 parts of pyrithiamine to one of thiamine, the effect develops. As shown in figure 11 pyrithiamine differs from thiamine only in the substitution of a —C=C— group for the —S— group in the thiazole fraction.

According to Keys and coworkers (12) the urinary excretion of thiamine in human subjects is partly in the form of pyrithiamine and the excretions of pyrithiamine are more constant than the excretion of free thiamine on a given intake of the vitamin. They suggest that assay of pyrithiamine excretion may be a better criterion for establishing deficiency than the determination of thiamine itself.

Thiaminase

A disease known as Chastek's paralysis was first observed on the fo ranch of J. S. Chastek at Glencoe, Minnesota. Ten per cent of uncooke fish was used in the ration and on this diet the foxes developed a diseas resembling Wernicke's syndrome, a typical thaimine deficiency effect (13) Since cooking the fish eliminated the effect, it was soon suspected that th causative factor was probably an enzyme.

Wooley (14) and Sealock et al. (15) confirmed this viewpoint and reported on properties and method of action of the enzyme. It is a protei enzyme with maximum destruction of thiamine at pH 9.1 and a temperature of 60° C.

TABLE 26

Table of approximate equivalents of thiamine

1 milligram of crystalline vitamin B_1 hydrochloride	=	333 International units 166 Smith Curative units 666 Chase Sherman units 6666 Cowgill milligram-equivalents
1 International unit	=	0.5 Smith curative units 2.0 Chase Sherman units 20 Cowgill milligram-equivalents 3.0 micrograms thiamine hydrochloride
1 Smith unit	=	6.0 micrograms thiamine hydrochloride 2.0 International units
1 Chase-Sherman unit	=	1.5 micrograms thiamine hydrochloride 0.5 International units
1 Cowgill milligram-equivalent	=	0.15 micrograms of thiamine hydrochloride 0.05 International units

According to Krampitz and Wooley (16) the enzyme splits thiamine at the methylene bridge; then in the case of the enzyme extracted from carp it forms a derivative with the pyrimidine fraction which by a second reaction liberates free pyridine. According to Hennessy et al. (17) the thiaminase extracted from raw clams shows only the first cleavage, and they have reported the isolation of the pyrimidine derivative.

Until the discovery of the enzyme in raw clams there appeared little possibilibility of danger to human beings from this enzyme. Deutsch and Hasler (18) have reported it present in 15 out of 31 species of fresh water fish and absent in 9 species of marine fish. Furthermore since it is destroyed by cooking the chance of its presence in human dietaries is small even when fish is used in the diet. However human beings do eat considerable amounts of raw clams and Melnick et al. (19) studied the effect of such a diet ingredient on human thiamine supply. They found that 100 grams of raw clams contained enough of the enzyme to destroy 7.5 mg. of thiamine given as a test dose and 50 per cent of the thiamine in the basal diet. Purification of the carp enzyme has been described by Agren (20).

In 1944 Bhagvat and Devi (21) noted that certain cereals, legumes and oil seeds inactivate added thiamine. The properties of the destructive factor indicate that it is not an enzyme and hence not thiaminase. The factor was extractable with water and largely stable to heat, also it was not affected by acid or alkali. Its nature is still undetermined.

Another substance antagonistic to thiamine has been reported present in certain poisonous ferns (22).

Expression of Thiamine Potency

Various methods of expressing thiamine potency are given in the literature, but with the present availability of the pure vitamin the preferred expression today is actual weight, in milligrams or micrograms. Table 26 shows the equivalents of the units used in the past with actual weight of thiamine.

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Section III. Evaluation of Thiamine in Human and Animal Nutrition

For therapeutic use the Council on Pharmacy and Chemistry of the American Medical Association (1) lists the following:

1. Thiamine is of value in correcting and preventing beri-beri.

2. Thiamine may be cited as of value in correcting and preventing anorexia of dietary origin in certain cases. (When the condition to be treated is due to deficiency of that vitamin.)

3. The administration of thiamine in excess of that present in the ordinary diet may be advantageous when there are specific conditions indicating interference with proper assimilation of the vitamins.

4. While it has not been established that thiamine deficiency is the sole cause of conditions described as alcoholic neuritis, the neuritis of pregnancy and pellagra, there is some definite evidence of the value of this vitamin in the treatment of these conditions. Vague representations with respect to the value of thiamine in the treatment of other types of neuritis are not permissible.

5. Thiamine deficiency in animals is associated with dysfunction of the heart and the vascular system. Thiamine is effective in reestablishing the normal function of the cardiovascular system if the dysfunction was caused by thiamine deficiency. Evidence is lacking that thiamine is effective in any other type of heart disease. At times organic heart disease and beriberi heart co-exist; administration of thiamine is justified in such patients.

6. It appears that there is an increased requirement for thiamine when there is greatly augmented metabolism such as occurs in febrile conditions, hyperthyroidism, or vigorous muscular activity.

Appraisal of Nutritional Status

Jolliff and Most (2) have given the signs and symptoms that obtain in vitamin B_1 deficiency. They classify them under three heads:

1. The neurasthenic syndrome.

2. Signs and symptoms involving the nervous system including peripheral neuropathy, Wernicke's syndrome, Korsakov's syndrome.

3. Signs and symptoms involving the circulatory system.

These, however, are matters requiring expert diagnosis and treatment.

Beri-beri is of rare occurrence in the United States. The neuritis of pellagra is somewhat more common, especially in regions where pellagra is endemic. But for the average person it is the daily requirement of thiamine necessary to the utilization of his food calories that is of major concern and which created the movement for the enrichment of white wheat bread and flour and of corn meal. Consequently there has been extensive research to determine just what is the human daily requirement of thiamine.

Estimates of Normal Thiamine Needs

The earliest attempt to reduce thiamine requirement to a mathematical formula was the study of Cowgill (3) already explained in Section I (See p. 104). This was based on calorie intake and body weight. The formula did not provide any factor of safety to cover variations in absorption and utilization in normal individuals. Cowgill's formula, applied to a 150 lb. adult man consuming 2400 calories per day, called for .767 mg. of thiamine per day.

For label control on thiamine products the Food and Drug Division of the Federal Security Administration (4) put the average minimum daily adult requirement at 1.0 mg. of thiamine.

The Food and Nutrition Board of the National Research Council (5) has published a set of suggested allowances for human beings. These are allowances and not specific requirements. In the original publication the Board calculated the need to be 0.6 mg. of thiamine per 1000 calories of diet intake. In their 1945 revision they lowered the allowance to 0.5 mg. per 1000 calories up to a total of 3000 calories, and 0.3 mg. of thiamine for each 1000 calories over 3000. They applied this rule regardless of sex, size or weight; take into account only calorie intake. In 1948 these allowances were further modified and the N.R.C. Allowances are shown in Table 27.

Methods of Estimating Thiamine Adequacy

Unlike vitamin A, thiamine is not stored to any extent in the body; the daily supply must be renewed every 24 hours or deficiency results. Since it is excreted in the urine, measurement of urinary excretion on a given intake of thiamine was taken as one means of arriving at the minimum requirement.

Urinary Excretion Tests

In 1946 Gifft and Hauck (6) reported variations of this test on four individuals based on four different criteria, viz. a) the basal 24 hour excretion, b) Percentage of intake excreted, c) Response to a 5 milligram oral dose, d) Response to a 1 milligram intramuscular dose. By any of these criteria there was some disappointment in the results which caused the investigators to comment:

> "The apparent disagreement in results observed when nutritional status was judged by these four criteria suggests that the range of normal values for these tests has not yet been satisfactorily established . . . more data are needed to establish the range of normal values for these urinary excretion tests and their relative sensitivity."

TABLE 27

Suggested daily thiamine allowances

(Food and Nutrition Board, National Research Council, 1948)

Subjects	Calories	Milligrams of thiamine
Man (156 lbs.)		
Sedentary	2400	1.2
Moderately active	3000	1.5
Very active	4500	1.8
Woman (125 lbs.)		
Sedentary	2000	1.0
Moderately active	2400	1.2
Very active	3000	1.5
Last half pregnancy	2400	1.5
During lactation	3000	1.5
Children up to 12 yrs.		
Under 1 yr	110/2.2 lbs.	0.4
1–3 yrs	1200	0.6
4–6 yrs	1600	0.8
7–9 yrs	2000	1.0
10–12 yrs	2500	1.2
Boys and Girls over 12 yrs.		
Boys		
13–15 yrs	3200	1.5
16–20 yrs	3800	1.7
Girls		
13–15 yrs	2000	1.3
16–20 yrs	2500	1.2

In 1939 Melnick et al (7) also reported on the dietary adequacy of thiamine using the urinary excretion criteria. At that time they reported finding the average 24 hour excretions of 15 normal men and 9 normal women to be 198 and 92 micrograms respectively; also that 14 per cent of an oral 5 mg. test dose was recovered from the men in 24 hours; 12 per cent from the women. Later, however, Melnick (8) modified his procedure and standards with these results:

First, the distinction between men and women can be eliminated because when the two sexes were on comparable dietary intakes the urinary excretions were similar.

Second, that a small parenteral dose (0.35 mg. per square meter of body surface) is preferable to the 5 mg. oral dose since it tends to eliminate variations in rate of excretion and variations in degree of intestinal absorption.

Keys et al. (9) have also reported a study of the urinary excretion data used for estimating thiamine deficiency. Like Gifft and Hauck they found striking variations in the responses of individuals, a difference not due to variations in fecal elimination, physical activity of the subjects, basal metabolism or body weight. They did suggest that measurement of pyramin (pyrithiamine) excretion might be a more accurate test than measurement of thiamine excretion itself.

On the basis of his tests Melnick put the critical excretion of urinary thiamine at 75 mcg.; his normal subjects excreted in excess of 50 mcg. in 24 hours. Keys et al used two groups (6 each) of normal men on 1 and 2 mg. of thiamine per day. The group on 1 mg. ranged from 32 to 91 mcg. of thiamine per day; average 62; the group on 2 mg. ranged from 86 to 810 mcg. per day, average 161 but with no correlation with stool content. The variations in pyramin excretions were much less and while only a small part of the thiamine excretion is in this form they suggest that its measurment may be a more accurate means of estimating thiamine need.

There have been other studies of this method, but until the causes for individual variations are determined the test is lacking in satisfactory accuracy.

Estimate by Blood Content

Thiamine, after absorption from the gut, is either stored as cocarboxylase or circulated as free thiamine in the blood. The blood content is measurable and has been used to estimate thiamine requirement and deficiency (See Table 28). Goodhart (10) has reported the normal blood content range to be from 3.1–9.2 mcg. per 100 ml.; that a content below 3 mcg. per 100 ml. indicates thiamine deficiency. As will be seen by reference to Table 28 the reported values are in fair agreement but all report that as an indicator of dietary deficiency they do not correlate well with intake in fact, they are less satisfactory than the urinary excretion data.

According to deJong (4), blood thiamine is distributed between the plasma and the blood cells; free thiamine in the plasma, cocarboxylase in the cells. He found 0.1 mcg. per 100 ml. of plasma and 5–11 mcg. of tota thiamine per 100 ml. of blood. The amount was affected by diet but als by muscle content.

It is also reported that blood levels do not respond to test doses as we as do urinary excretions. Youmans (12) has noted that, while the bloo level is lowered in severe deficiency in normal individuals, increased intak does not cause a rise in blood level.

There is, then, general agreement with Van Veen (13) who considers bloo level values of little significance compared with urinary excretion value in detecting dietary inadequacy or estimating thiamine needs.

TABLE 28

Reported normal blood thiamine levels

Year	Investigators	Milligrams per 100 ml. of blood	
		Range	Average
1938	Schneider and Burger		6.4
1938	Rowlands and Wilkinson	6.5–16.5	
1939	Widenbauer et al.	2–11	
1939	Sinclair	5.5–10.5	7.4 ± 1.4
1940	Magyar		7.6
1941	Lehman and Holger	7–14	9
1941	Goodhart and Nitsberg	3.1– 9.2	
1942	Deutsch	9–16	
1942	Costoya	1.4–24.8	15.0
1943	Friedemand and Kimieciak	3–11.2	5.7
1944	Oldbeauer et al. (in 5 yr. old children)	4.9– 7.5	

Measurement of Blood Pyruvate Content

In thiamine deficiency there is an accumulation of pyruvate in the tissue and an increased flow from the tissues into the blood. Bueding and Wortis (14) have developed a satisfactory test for measuring blood pyruvate content and this has been suggested as a means of detecting thiamine deficiency or need. Wilkins et al. (15) have criticised it as nonspecific but the main objection seems to be that values are small except in severe grades of deficiency.

Jolliffe (16) however, states that in thiamine deficiency it is logical to expect pyruvemia and the test is worth study. He has reported on the pyruvate blood content in normal subjects and in those with a variety of illnesses; also results when under metabolic strain produced by the ingestion of 1.75 grams of dextrose per kilo of body weight. He found an average of 1.0 mg. per cent and a range of 0.77–1.16 mg. per cent in 87 normal subjects. He puts the upper normal limit at 1.3 mg. per cent.

In 27 normal subjects pyruvemia was measured for 5 hours following glucose injection. The maximum varied from 0.14 to 0.93 mg. per cent and averaged 0.43 mg. per cent above the fasting level. This level was reached at the end of one hour in every case but one (that one in 30 minutes). Following arrival at the peak, it fell and reached the fasting level in the third or fourth hour. In thiamine deficiency subjects the fasting level was elevated in 45 out of 48 subjects.

Blood pyruvate measurements may, then have value, especially for detection of thiamine deficiency.

Summary of Thiamine Requirement Estimates

In Table 29 from Melnick (17) is shown a comparison of reported estimates of minimal thiamine requirements for human subjects. Holt (20) put his estimate still lower than shown in Melnick's Table. On a constant diet he finds the minimum for the normal adult to be 0.13–0.17 mg. per 1000 calories. A range between 0.24 and 0.44 mg. per 1000 calories was protective against thiamine deficiency. He also claims that this estimate, based on calorie intake only, holds for all ages. Incidentally neither Elsom (19) nor Najjar (24) found any correlation between the thiamine requirement and body weight. All agree that increasing the fat calories in the diet tends to spare the thiamine requirement.

Intestinal Synthesis of Thiamine

It has been known for some time that ruminant animals such as cows and ewes are practically independent of dietary supply of thiamine owing to the fact the vitamin is synthesized by bacteria in their rumen in sufficient quantity to meet their needs. Rats also synthesize it in the gut and void such synthetic vitamin in their feces making it necessary to avoid coprophagy when using rats in assaying food vitamin content. They do not however absorb enough of such synthesized thiamine to meet their needs.

Najjar and Barrett (24) have summarized evidence that human beings also get synthetic thiamine formed by their intestinal bacteria and can absorb the vitamins so formed. Such supplies would tend to change the results of test doses if the amount of the absorbed synthetic vitamin was of significant amount, but it is generally agreed that human beings get very little of such supply.

Incidentally, while it is true that there is little storage of thiamine in the body compared with the storage of vitamin A and that a new supply is needed in the diet daily to meet requirements, it is also true that there is

TABLE 29

A critique of values suggested as the thiamine requirement of man

(After Melnick (17) 1944)

Year of report	Investigators	Test subjects	Nutritional status of the subjects	Type of experimental observations	Daily minimal requirement per 1000 calories	
					Found	Recommended
					mg.	mg.
1941	Williams and Mason (29)	11 sedentary female students	Partial thiamine depletion	Recovery from subjective symptoms of poor health		0.5–1.0
1942	Elsom, Reinhold, Mickelsen, Chorneck (30)	6 sedentary adult women	No preexisting signs of deficiency	Minimal intake compatible with health	0.35	
1942	Melnick (8)	116 sedentary adults, men and women	60 well nourished as determined by clearance test; 50 undernourished on prolonged deficiency	Thiamine clearance tests before and after saturation. Determination of critical level of thiamine intake with precipitous drop in urinary thiamine	0.35	0.5
1942	Holt (31)	12 sedentary adult men	Presumably well nourished	Minimal thiamine intake for fasting to be zero	0.26–0.31	
1943	Keys, Henschel, Mickelsen and Brozek (9)	10 active adult men	Well nourished. In robust health	Ability to do severe work; tasks of psycho-motor function to maintain normal carbohydrate function, to show variation in urinary excretion	<0.23	
1943	Williams, Mason and Wilder (32)	11 sedentary adult women	Well nourished	Critical level associated with urinary excretion and biochemical defect in carbohydrate metabolism	0.45	0.6

some storage in the body organs and Taylor et al have compiled the data shown in Table 30 which shows that heart, liver and kidney are the major storage depots.

Bread and Flour Enrichment with Thiamine

In view of the importance of thiamine for utilization of carbohydrate and for prevention of beri-beri following a too-exclusive use of carbohydrate, it was a logical step to consider fortification of carbohydrate foods such as white flour, white bread and corn meal with the vitamin. This was especially necessary since these staple foods form so large a part of the fuel foods of the people.

TABLE 30

Tissue storage of thiamine

(After Taylor, Pollack, and Williams (26) 1943)

Organs	Micrograms thiamine per gm.
Heart	3.0 –4.5
Liver	2.0 –2.5
Kidney	2.4 –3.6
Skeletal muscle	0.84–1.5
Smooth muscle	1.2
Brain	1.4 –1.8
Lung	0.6 –1.6
Ovary	0.55–1.0
Testes	0.42–0.63
Skin	

Note: Major storage is in heart, liver, and kidney.

During the War years a plan for a suitable enrichment of white bread and white flour was worked out and the enrichment of white bread made compulsory throughout the United States. The basis of this enrichment was not, as many believed, to *restore* bread and flour to the nutrient value of whole wheat bread and flour. On the contrary it was built on what was called the "*carry its share*" plan. In brief, that meant to add enough thiamine to insure the utilization of the calories supplied by the bread or the flour (See Table 31). As a matter of fact if we take 0.5 mg. of thiamine per 1000 calories as the requirement the actual enrichment supplied not only the actual need but a generous factor of safety.

The War order that made enriched bread compulsory expired on October 18, 1946, but since that date and at this writing 19 States, Porto Rico and Hawaii have passed legislation making enriched bread compulsory within their boundaries.

In view of the extent of pellagra in certain sections of the country and its relation to corn meal in the diet supplementing has also been suggested for this staple carbohydrate and is now in operation in certain regions.

TABLE 31

Comparison of the thiamine content of enriched and unenriched white bread and flour

Products	Calories per lb.	Mg. thiamine per lb.	Thiamine required @ 0.5 mg./1000 calories	Factor of safety
Bread (unenriched)	1185	0.31	0.593	None
Bread (enriched)	1185	1.00	0.593	30%
Flour (unenriched)	1615	0.23	0.808	None
Flour (enriched)	1615	1.66	0.808	50%

Converted Rice

Still another movement in the direction of improving public health has been the enrichment of polished rice. For some time it has been recognized that the beri-beri endemic in the Orient was the result of a too exclusive white rice diet and it was also known that the substitution of brown rice would eliminate the menace, but as in the case of whole wheat and white flours popular preference is for the polished rice.

Two methods of making white rice richer in thiamine have been evolved (27). In 1932 Akroyd (28) showed that if rice is parboiled before milling, a considerable part of the thiamine in the hull and polish is diffused into the endosperm. In fact, Akroyd and coworkers (29) reported that parboiled rice, even when highly milled, retains most of the thiamine and niacin present in the unmilled grain and only about 50 per cent of the vitamins are lost in washing and cooking.

The crude processes of parboiling and milling used in certain parts of India have been modernized and today the product in the American market known as converted rice is the result. Converted rice is made by cooking unhulled rice under pressure, then drying and milling. In this process the starch is gelatinized, the B vitamins diffuse into the endosperm and the product retains nearly 68 per cent of the original content of the rice before treatment.

In addition to converted rice there is also available today an enriched rice made by impregnating a fraction of polished rice with a vitamin concentrate and then diluting this fraction with untreated white rice to the desired concentration. By one patented process the vitamins are supplied in a glucose-dextrin solution.

Increased use of such products should go far to reduce the danger of beri-beri and other vitamin B_1 deficiency effects.

Thiamine in Live Yeast

Yeast was early found to be exceptionally rich in thiamine. It was also found possible, by incorporating certain chemicals in the culture medium, to enhance the normal content of the vitamin. As a result dried yeasts, especially brewer's yeasts, have had extensive therapeutic use as rich sources of thiamine. At one time the use of live yeasts, such as found in baker's yeast cakes, were advocated to supplement the diet. Recent studies have, however, shown that live yeasts when swallowed do not necessarily give up their vitamin for human absorption. Kingsley and Parsons (30) have reported that the thiamine in unfortified fresh baker's yeast when ingested by humans is unavailable for absorption. Not only does the yeast not give up its thiamine but it also competes with the body for the thiamine in the diet. If baker's yeast is first killed by commercial drying processes or by treatment with boiling water no such effect results; only the live yeast is unavailable as a source of thiamine.

Animal Requirements for Thiamine

All animals require thiamine, but not always as a dietary ingredient. Cattle over two months of age synthesize thiamine in the rumen in amount ordinarily sufficient to meet their needs. It is therefore rarely necessary to incorporate extra thiamine in cattle rations. The same process occurs in the sheep. It is possible however for this supply to fail if, through other deficiencies in diet, the bacteria that form the thiamine in the rumen do not get suitable materials for their activity. Until the rumen supply is established the calves also may need thiamine supplement. Ordinary cow's milk averages about 0.32 mg. of thiamine per quart.

Growing pigs require about 2.5 mg. of thiamine per day per 100 pounds live weight. Pregnant gilts, sows and young boars require 3.0 mg. per day and lactating sows 6.0 mg. per day. Either composition or fortification of diets should supply these amounts.

The recommendations for chicks are 0.9 mg. per pound of feed. Mature fowls require it and suffer from deficiency, but the amount necessary has not been accurately established.

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CHAPTER VIII. RIBOFLAVIN OR VITAMIN B_2 OR G

SECTION I. FUNCTIONS OF RIBOFLAVIN

As stated in the history of thiamine development, the existence of vitamin B_2 was postulated when water-soluble B was shown to contain at least two growth-promoting factors, one more heat stable than the other (1, 2). At one time it was suggested that the heat stable factor be called vitamin G and the heat labile factor vitamin F. Eventually however, the English choice of B_1 for the heat labile and B_2 for the heat stable factor became generally adopted. In the United States B_2 is still some times called vitamin G.

The growth-promoting effect was the earliest function established for vitamin B_2 and the first assay method of Bourquin and Sherman (3) was based on its growth promoting effect in rats. At that time the vitamin had not been isolated but Booher (4) reported extracting from whey a yellow pigment that gave the Bourquin-Sherman test response.

As far back as 1879 Blyth (5) reported the presence in cow's milk of a yellow pigment but gave no data on its possible functions. Bleyer and Kallman (6) in 1925 also described the pigment as a hitherto little-studied substance in cow's milk.

The Yellow Ferment

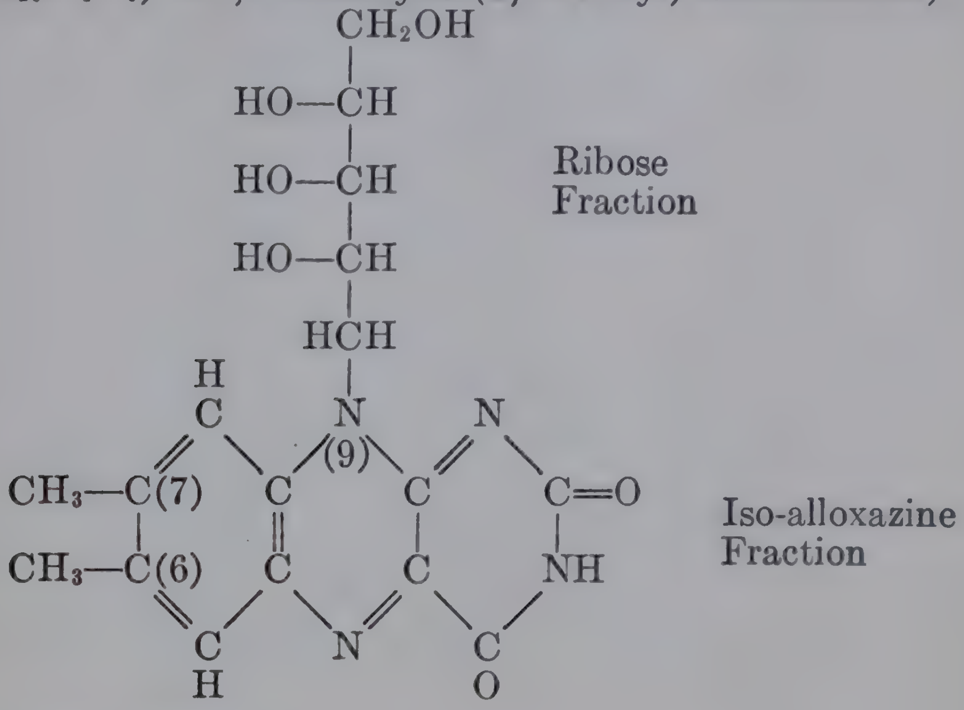
In 1932 Warburg and Christian (7) reported the discovery of a new oxidation enzyme present in bottom yeast and tissue extracts. On account of its yellow color and green fluorescence in water solutions they called it the yellow ferment, or yellow oxidation enzyme. This yellow enzyme, in combination with diphospho-pyridine nucleotide as coferment, caused the oxidation of glucose-monophosphate.

Gyorgy (8) has described the research which led to the identification of the colored component of the yellow enzyme with vitamin B_2. The isolation was accomplished in 1933 by Gyorgy in collaboration with Kuhn and Wagner-Jauregg (9). The term "flavin" was used to indicate the pigment and originally it was prefixed with a source name (lacto-, ovo-, hepato- etc.) to indicate the material from which it was extracted. Ultimately it was shown that all these flavins of different origin were identical structurally, namely a derivative of iso-alloxazine with two methyl groups and a pentose sugar radical attached. The pentose sugar was ribose and the name riboflavin was formally adopted by the Council of Pharmacy and Chemistry of the American Medical Association in 1937. Several laboratories participated in its chemical identification (11, 12, 13). For structure see figure 12.

The yellow ferment itself consisted of a phosphorylated riboflavin (cytoflav) attached to a protein. It acts as a dehydrogenase but in order to effect dehydrogenation, a coferment is necessary and two such were identified as

A. Riboflavin Structure

($C_{17}H_{20}N_4O_6$) or 6,7-dimethyl-9-(d,1′-ribityl) iso alloxazine).



B. The Yellow Ferment

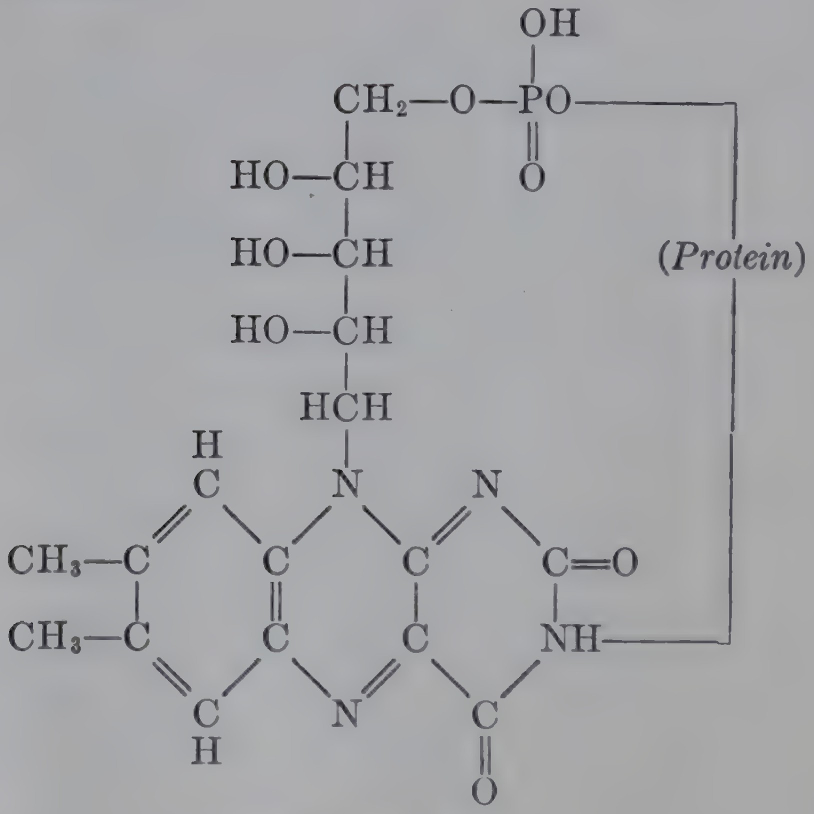


FIG. 12

C. A Yellow Ferment Co-ferment
Diphospho-pyridine Nucleotide

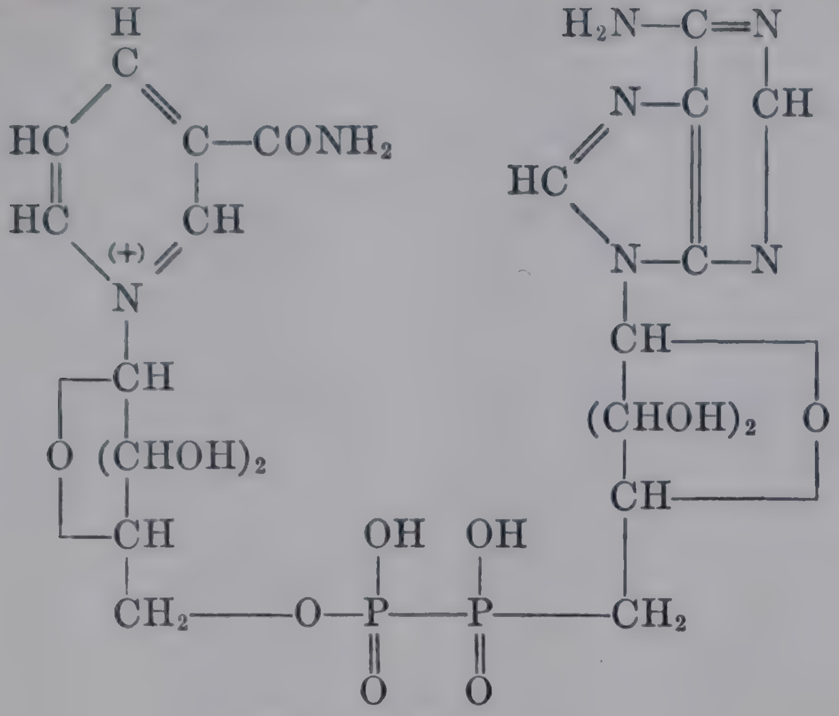


FIG. 12—*Continued*

di- and tri- phospho pyridine nucleotides. In these the hydrogen transferring group of the coferment is nicotinic amide (See figure 13).

Riboflavin is apparently needed by all animals and certain microorganisms. Lack of it manifests itself first by retardation and later by complete cessation of growth. One of its basic functions, then, is growth control and nutrient metabolism.

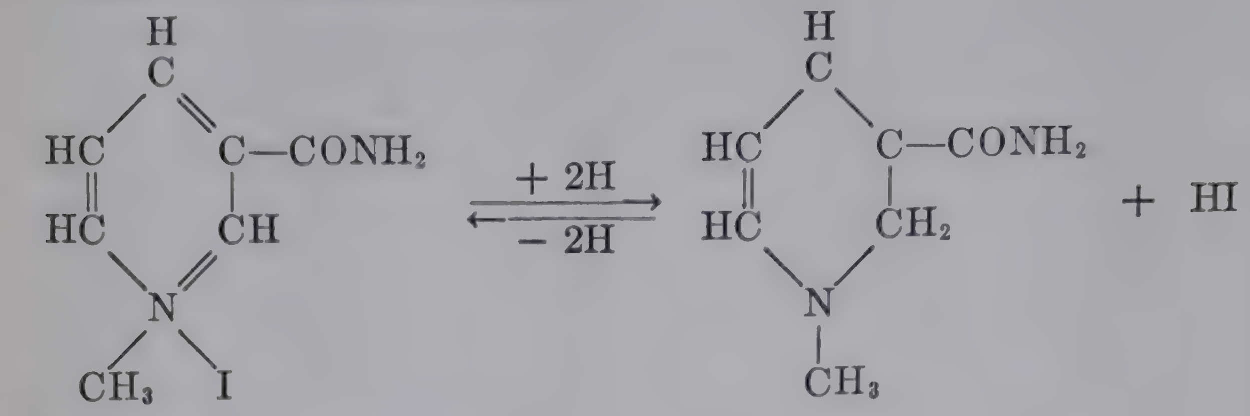
But, as in case of thiamine deficiency, inadequacy of riboflavin in the diet may also manifest itself in other ways than in growth control or weight maintenance.

To date several flavo-proteins have been identified and Table 32 by Hogness (14) shows what these are and their characteristics.

Rat Pellagra

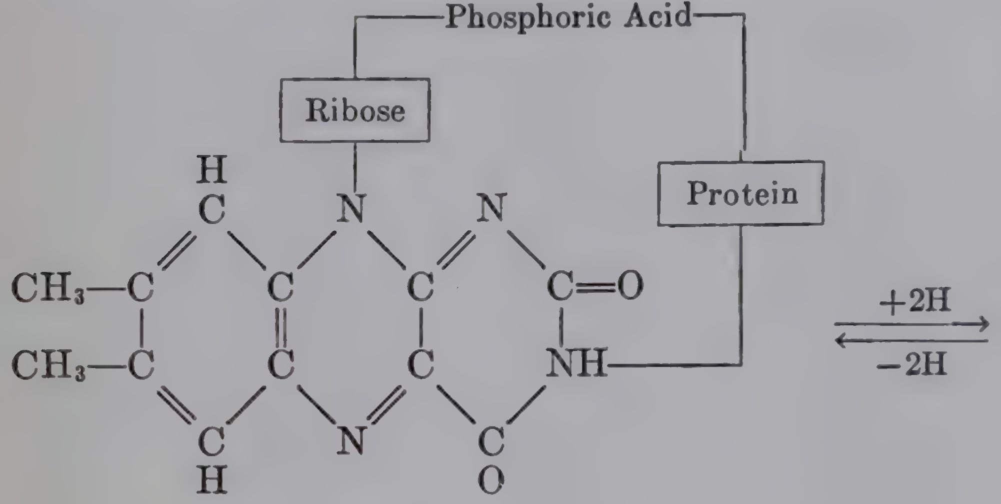
In the early days of the study of the heat stable fraction of water-soluble B it was noted that when the factor was absent from the diet not only was growth affected but a dermatitis developed. In some respects this dermatitis resembled the lesions of human pellagra. This led to the postulation by Goldberger et al. (25) that the vitamin was the anti-pellagric factor. Later however the B_2-deficiency dermatitis was shown quite distinct from that of pellagra or of its analogue, the black tongue of dogs, (26, 27, 28, 29, 30). Out of these studies came the later discovery of vitamin B_6, or pyridoxine, and still later the actual isolation of the true antipellagric vit-

A. Behavior of the nicotinic acid:

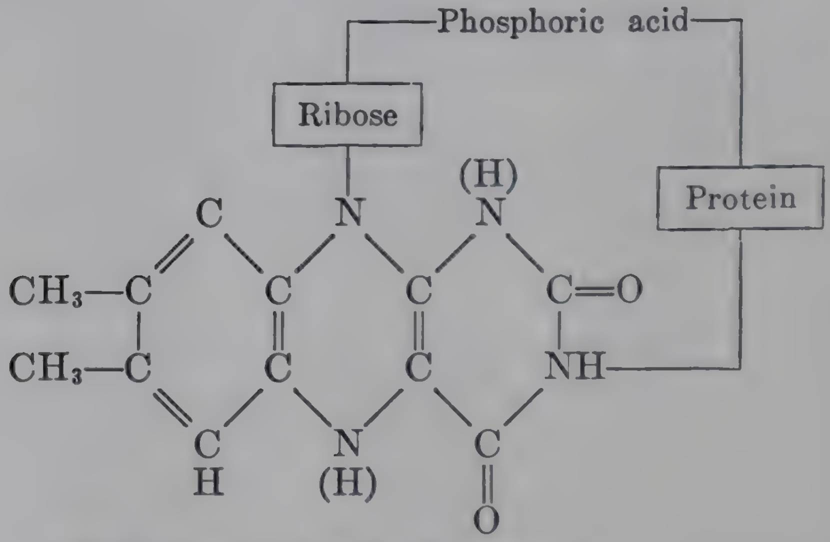


Metiodide of Nicotinamide
Oxidized form

B. Behavior of the Yellow Enzyme:



Yellow Enzyme



Hydrogenated Yellow Enzyme
(Leuco form)

FIG. 13. MANNER IN WHICH THE NICOTINIC AMIDE OF THE COFERMENT AND THE ISOALLOXAZINE FRACTION OF THE YELLOW FERMENT TRANSFER HYDROGEN

(After Schopfer, Plants and Vitamins, Chronica Botanica Co.,
Waltham, Mass., (1943)

TABLE 32

Flavo-proteins and their characteristics

(After Hogness (14) 1942)

Enzymes	Identified by	Reference	Source	Prosthetic group	Reducing systems	Oxidizing systems
Original yellow enzyme	Warburg and Christian (1932)	(7)	Yeast	Riboflavin phosphate (cytoflav)	Tri- and di-phospho-pyridine nucleotides	Oxygen, cyto-chrome c
d-amino acid oxidase	Krebs (1935) Warburg and Christian (1938)	(15) (16)	Kidney cortex	Alloxazine adenine di-nucleotide	d-Amino acids except d-glutamic	Oxygen
New yellow enzyme	Haas (1938)	(17)	Yeast	Same as above	Di-phospho-pyridine nucleotide	Methylene blue oxygen
Straub's yellow enzyme	Straub (1939)	(18)	Heart muscle	Same as above	Same as above	Methylene blue oxygen
Crossed yellow enzyme	Warburg and Christian (1938)	(19)	Yeast	Same as above	Tri- and di-phospho-pyridine nucleotides	Oxygen
Xanthine oxidase	Ball (1939)	(20)	Milk	Same as above	Xanthines, other purines aldehydes di-phospho pyridine nucleotides	Oxygen
Fumaric dehydro-genase	Fischer and Eysenbach (1937) Fischer et al. (1939)	(21) (22)	Yeast	Same as above	Reduced dyes	Fumarate, malate, etc.
Aldehyde oxidase	Gordon, Green and Subrahmanyan (1939)	(23)	Liver	Same as above	Aldehydes	Oxygen, methylene blue
Cyto-chrome C reductase	Haas, Horecker and Hogness (1940)	(24)	Yeast	Riboflavin phosphate	Tri-phospho pyridine nucleotide	Cyto-chrome c, oxygen

amin now known as nicotinic acid or niacin. However, skin and other lesions are producible by vitamin B_2 deficiency.

Some Manifestations of Riboflavin Deficiency

In 1938 Sebrell and Butler (31) reported a study of human subjects on a riboflavin deficient diet. These subjects developed in the corners of the lips macerated areas which progressed to transverse fissuring (cheilosis). The labial mucosa became shiny, abnormally red, and denuded. Greasy, seborrheic accumulations were observed around the nose and eyes and in some cases the ears. Oral administration of riboflavin resulted in relief.

In 1939 Bessey and Wolbach (32) noted that corneal vascularization was the first morphological manifestation of riboflavin deficiency in rats. Kruse et al. (33) described the same effects in man accompanied by photophobia, dimness of vision, burning sensation in the eye, soreness and swelling of the eyelids and impariment of visual acuity.

In the search for specific diagnostic signs to show riboflavin deficiency these manifestation have received intensive study. To date, while it is agreed that these changes can result from riboflavin deficiency it is also true that similar changes can be produced by other causal agents. In brief, the signs lack true specificity, and cannot be accepted as definite signs of B_2 deficiency without further check to eliminate other potential factors.

Cheilosis and Tongue Changes in Riboflavin Deficiency

According to Sydenstricker (34), cheilosis begins as a tiny, red, painful area in the corners of the lips at the muco-cutaneous junction and may progress to painful fissuring. It cannot be considered specifically characteristic of riboflavin deficiency because its presence may depend upon concurrent deficiencies, vitamin B_6, niacin, the entire B-Complex, or iron. To this list of possible causes Jeghers (35) adds the effects of lipstick, dental plates, chewing gum, tooth pastes, mouth washes, cigarette holders, throat lozenges, reeds of musical instruments and even sun exposure. In brief, cheilosis can result from riboflavin deficiency but presence of cheilosis is not definite evidence of the deficiency (36, 37, 38, 39, 40). Its appearance justifies treatment by riboflavin therapy but only response to that therapy justifies the assumption that the vitamin deficiency is the causal factor in any given case.

Jeghers (41) has described in detail the tongue changes produced by ribiflavin deficiency. According to him:

> "Epithelium over the papillae does not desquamate, but appears flattened and edematous, with resultant mushroomed shaped papillae that give to the surface of the tongue a finely pebbled or granular appearance. The tongue may be painful, may have a burning sensation, and may even cause difficulty in swallowing. These tongue changes may precede and almost constantly accompany the cheilosis and seborrheic dermatitis of the deficiency state."

He explains the magenta coloring of the tongue that has been described as characteristic of this deficiency as follows:

> "Capillary dilatation and proliferation are prominent features in B_2 deficiency and undoubtedly take place in the mucosal portion of the papillae. If the capillaries are dilated, one must postulate that the circulation of red corpuscles through them must be slowed. The overlying epithelium is not desquamated but thinned and perhaps edematous. These dilated capillaries with their stagnant blood flow seen through the changed epithelium give the resultant magenta color to the tongue. The rapid blanching of the tongue following riboflavin therapy further supports this explanation."

Ocular Changes in Riboflavin Deficiency

The statement of Bessey and Wolbach (32) that corneal vascularization is the first morphologic mainfestation of B_2 deficiency in rats has already been cited. The American Medical Association's Council on Pharmacy and Chemistry, in giving allowable claims for riboflavin therapy puts the statement as follows:

> "Riboflavin deficiency is responsible for certain ocular manifestations characterized by itching, burning and a sensation of roughness of the eyes (keratitis), accompanied by mild photophobia. The anatomical changes may vary from a superficial invasion of the cornea by capillaries to an extensive vascular proliferation, with or without infiltration, opacity, and exudate formation. These symptoms, *when due to a riboflavin deficiency*, are relieved promptly by the administration of adequate amounts of riboflavin."

Corneal vascularization, detectable by the slit lamp, held great promise of being a specific means of diagnosing B_2 deficiency. Kruse et al. (42) and Wiehl and Kruse (43) held from extensive examinations of subjects that if corneal vacularization and cheilosis are legitimate evidence of B_2 deficiency, it is probably the most common vitamin deficiency in the United States today. However, as in the case of cheilosis, evidence has accumulated to show that these eye changes can result from B_2 deficiency but *are not specific evidence of such deficiency*. Vail and Asher (44) reported that any form of inflammation of the conjunctivae may lead to congestion in the meshwork about the cornea. To regard this reaction as a specific form of B_2 deficiency is unjustified. Lysine and tryptophane deficiency (45, 46, 47), zinc deficiency (48), and the effect of thallium (49) have all been cited as producing similar eye effects.

In brief, eye changes detectable by evidence of corneal vascularization are today not acceptable as definite proof of B_2 deficiency but are producible by such deficiency. When so produced they respond to riboflavin therapy. It is also evident that this type of deficiency effect is fairly common (50).

While ocular, skin and tongue manifestations of B_2 deficiency have been the principal changes reported and intensively studied, they are not the only effects that have been attributed to B_2 deficiency. It has been claimed that such deficiency can produce blood dycrasias.

Effect of B_2 Deficiency on Blood

Waisman (51) in reporting on the effect of riboflavin deficiency in the monkey, noted anemia as one of the results. Erythrocyte levels and hemoglobin levels fell to the anemic stage shortly after the appearance of the dermatitis. Gyorgy et al. (52) reported that riboflavin therapy increased hemoglobin formation in an anemic dog. In contradiction Sebrell and Onstott (53) found anemia frequent in B_2-deficient dogs but not responsive to riboflavin therapy. Patek et al. (54) noted development of anemia in B_2-deficient pigs but attributed it to lack of other food factors and Keys et al. (55) failed to get any signs of anemia in young men held for 84 days on a low B_2 intake (0.31 mg. per 1000 calories daily). Kornberg et al. (56) have reported agranulocytopenia in rats on a B_2-deficient diet.

Shaw and Phillips (57) found that rats in severe B_2 deficiency showed partial paralysis of the legs, a condition more easily produced on a high fat ration. The nerve changes were characterized by degeneration of the myelin sheaths and accompanied by axis cylinder swelling and fragmentation. They also observed myelin degeneration and gliosis of the spinal cord. Bethke and Record (58) claim riboflavin specific for preventing curled toe paralysis in chicks; that while 250 γ per 100 grams of ration is adequate for maximum growth in 8 weeks it requires 300 γ per 100 grams of ration to prevent the curled toe effect.

Effects Related to Riboflavin as Enzyme

Gyorgy (8) has stated:

> "Deficiency of riboflavin must naturally lead to a disruption of all cellular reactions that are linked with enzyme processes in which flavo-protein participates. From the nutritional point of view, one should, however, bear in mind the important fact that in addition to lack of riboflavin, lack of substrate will have an equal effect on cell metabolism and therefore also in respect to pathological manifestation."

In illustration of this point he cites the relation of riboflavin to the prevention of liver injury following ingestion of butter yellow. Kensler et al. (59) reported that riboflavin was essential provided casein was present in the diet and that casein was replaceable by a combination of cystine and choline. The explanation was that in this case riboflavin in the enzyme reacts with cystine and choline to produce methionine which is probably actually responsible for the protection.

He might also have cited the relation of riboflavin to the control of estrus. The Biskinds and co-workers (60) in 1941 and 1942 pointed out that one function of normal livers is the inactivation of the estrogen hormones that produce estrus and that the ability of the liver to do this is decreased by a deficiency of B-Complex vitamins in the diet, notably of vitamins B_1 and B_2. Ashworth and Sutton (61) in the same year had noted that the administration of estrogen to persons showing subclinical B-Complex deficiency caused the appearance of lesions of B_1, B_2, and niacin deficiency, either increasing the demand for those vitamins or suppressing their utilization.

In 1944 the Segaloffs (62) showed that inactivation of estrone alpha-estradiol by rat liver is decreased in the absence of thiamine and ribo-flavin but not by absence of choline, calcium pantothenate or pyridoxine. In the same year Singher et al. (63) made an *in vitro* study of liver slices from rats on B_2- and B_1-deficient diets and from controls on the same basal diet but plus adequate B_1 and B_2. In this study the slices of liver from the B_1- and B_2-deficient rats failed to inactive estradiol while those from the controls showed this power. They found the critical liver content of B_2 to be 14 γ per gram. They also reported that B_1 and B_2 could not be substituted for by B_6, pantothenic acid, or vitamin A.

But, as in the butter yellow experiment, the level of protein in the diet affected the liver behavior and the ability to store B_2. Sarrett and Perlzweig (64) had already shown that the concentration of B_2 in liver varied with the level of protein in the diet and was independent of the intake. That the effect of a low protein diet could be partly compensated by supplementing the diet with methionine and cystine.

In these two examples we have evidence of the effect of B_2 on a tissue function which is accompanied by no lesions but shows itself in failure of the organ to perform normally. In this type of action the vitamin acts as part of an enzyme oxidation system requiring, in addition to the vitamin, certain subtrate materials for the reaction.

Effect of Diet Components on Riboflavin Requirement

In the cases just cited there was a relation of the B_2 requirement to the amount of protein in the diet. It has also been demonstrated that other dietary ingredients can affect the B_2 requirement. Mannering et al. (65) showed that when dextrin or cornstarch forms the carbohydrate of the diet rats needed less riboflavin. Substitution of other calorie food such as sucrose, cellulose, or lard did not have this effect. In these experiments the carbohydrates that decreased the B_2 requirement increased its intestinal synthesis which in this case may be the explanation: with greater intestinal synthesis less of the vitamin was needed to supplement the diet.

While, unlike vitamin B_1, vitamin B_2 does not stimulate appetite it does have a value for utilization of the food (66).

Riboflavin and Cataract

The effect of B_2 deficiency on eyes has been already discussed. In 1931 Day et al (66) reported that B_2 deficiency in rats could produce cataracts and later (67) that the same effect was producible by B_2 deficiency in mice, chickens and monkeys. It appeared that the cataracts could be prevented and relieved by adequate riboflavin administration, (69, 70, 71). Other investigators (32, 72, 73) could not confirm these findings. Galactose diets are also known to produce cataract (74), but this form does not respond to B_2 therapy. The value in human cataract has not been demonstrated.

Relation of Riboflavin to Infection

There have been reports tending to show that riboflavin deficiency lowers resistance to certain infections. Pinkerton and Bessey (75) reported that B_2 deficiency in rats greatly lowered their resistance to endemic typhus; Wooley and Sebrell (76) that mice fed a B_2-deficient diet succumbed more readily to the intraperitoneal injection of virulent pneumococci. On the other hand Seeler and Ott (77) have shown the parasitization of the red cells in the blood of the chick by the malarial organism (*P. lophurae*) can be reduced by reducing the B_2 intake. Gyorgy (77, 8) found that his B_2-deficient rats developed louse infestation which responded to B_2 therapy.

The relation of these vitamins to infection needs further study. Schneider (79) has reviewed this problem and the precautions necessary to proof of specific vitamin action.

SUMMARY

The preceding section discusses some of the effects associated with riboflavin deficiency. It must be evident that while these effects are known to follow riboflavin deficiency their appearance is not per se, evidence of such deficiency. But as Sherman (8J) puts it:

> "While there may be a doubt as to the relations of riboflavin to disease, there is no doubt as to its importance to health, efficiency, vigor, and resistance."

Its value in human and animal nutrition is discussed in Section III.

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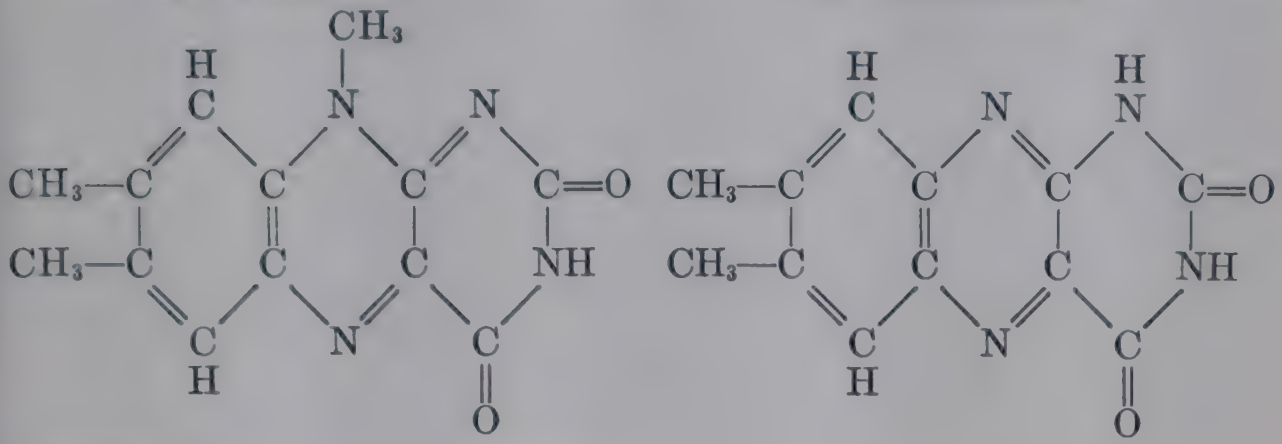
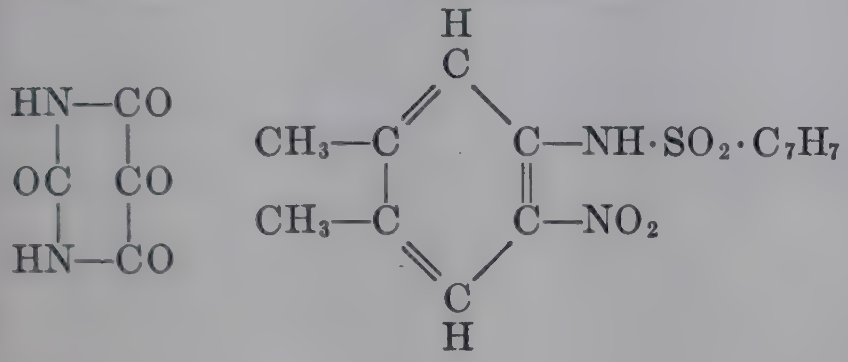
Section II. Forms and Chemistry of Riboflavin

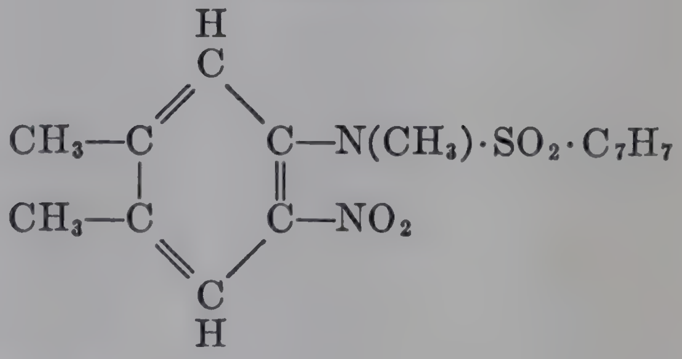
Booher (1) has described in detail the steps that led up to the chemical identification of riboflavin (See figure 12). In brief, Warburg and Christian (2) split their yellow ferment into a protein fraction and a pigment component. Irradiation of an alkaline solution of the pigment component followed by acidification of the irradiated solution yielded a yellow, chloroform soluble, photoderivative later named lumiflavin (See figure 14). Study of this photoderivative finally disclosed the chemical structure of the vitamin and determination of its relation to the alloxazine series of organic compounds first discovered by Kühling (3) in 1891 (See figure 14).



A. Lumiflavin B. Lumichrome



C. Alloxan D. Alloxazine



E. Isoalloxazine

FIG. 14

Kuhn et al. (4) showed that irradiation of alkaline solutions of riboflavin split off a hydroxyl-rich side chain later identified as the sugar ribose, with lumiflavin as the other fraction. Meanwhile riboflavin was isolated from egg white (5), milk (4), liver (6), kidney (7), urine (8), barley malt (9), dandelion blossoms (10), grasses (11), egg yolk (12) and the retina of fish eyes (13). These flavins were originally supposed different in structure and were identified by prefixing the term flavin with source name (lacto-, ovo-, hepato- etc.). Later they were all found to be of the same structure, ribose combined with lumiflavin.

In 1934 Kuhn and Rudy (14) suggested a structural formula for lumiflavin. Work in both Kuhn's and Karrer's laboratories made rapid progress. Lumiflavin was synthesized by Kuhn and Reinemund (15) in 1934 and riboflavin itself was synthesized by Kuhn et al (16) and by Karrer et al (17) in 1935.

One of the striking characteristics of riboflavin is its sensitivity to light. As already stated, if it is irradiated in alkaline solution, the main photoderivative is lumiflavin but another form known as lumichrome (see figure 14) is also produced. Irradiated in neutral solution the ribose residue is completely eliminated and lumichrome only is left.

To form the yellow enzyme, riboflavin is first phosphorylated to form cytoflav and this in turn is combined with a protein (See figure 12). Figure 13 shows how the nicotinamide of the coferment and the yellow enzyme transfer hydrogen when the system acts as an oxidation system. The transformation of riboflavin to flavin phosphoric acid is said to occur in the intestinal wall and also as a general cellular reaction and thus to be independent of the method of administration, whether orally or parenterally (18).

There have been many derivatives of riboflavin synthesized (1), but few alterations in structure have been found compatible with vitamin activity. Substitution of l-arabinose for d-ribose produces a compound with about one third the potency of riboflavin. At least one of the methyl groups in position 6 or 7 is essential, complete absence of both methyl groups at 6 and 7 is accompanied by toxicity (19). Alterations of the molecule by replacement of the methyl group at position 6 with one in position 5 or of replacement of the one at 7 by one at 8 destroys both the vitamin and coenzyme activity (20). As regards the side chain only d-ribose and l-arabinose have proven compatible with vitamin activity.

It will be recalled that substitution of a —C═C— group for the —S— group in thiamine forms a product antagonistic to thiamine (pyrithiamine). Similar substitution products of riboflavin have the same effect. Wooley (21) lists three, viz 2,5 diamino 7,8 dimethyl 10 ribityl 5,10 dihydrophenazine, 6,7 dichlor 9 ribityl isoalloxazine, and 5,6 di methyl 9 ribityl isoalloxazine. Galacto flavin has also been reported (22) to prevent growth and to be compensated by riboflavin.

Chemical and Physical Properties of Riboflavin

Riboflavin is sold today in the form of an orange-yellow crystalline powder which is practically odorless and with a bitter taste.

The empirical formula is $C_{17}H_{20}N_4O_6$; molecular weight 376.36.

The melting point is about 275° C. with decomposition.

Solubility: 12 mg. in 100 cc. water at 25° C.; 4 mg. in 100 cc. 95 per cent ethyl alcohol at 25° C.; slightly soluble in cyclohexanol and amyl acetate but insoluble in ether, chloroform, acetone, or benzene. pH of a saturated water solution is approximately 6.

The aqueous solution is pale greenish yellow and has an intense yellow green fluorescence, maximum between pH 6 and 7 and in 0.003 per cent solution.

Riboflavin is relatively heat stable, stable to air and oxygen. But, very sensitive to light. Also sensitive to alkalies but quite stable in strong solutions of mineral acids.

Riboflavin exhibits characteristic absorption bands with well defined maxima at 220, 267, 376, and 446 $\mu\mu$; extinction coefficient for wave length 267 is 3.4×10^4, for wave length 446 is 1.15×10^4.

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SECTION III. EVALUATION OF RIBOFLAVIN IN HUMAN AND ANIMAL NUTRITION

In Table 33 is shown the recommended daily allowances of riboflavin as first given in 1941 and as revised in 1948 by the Food and Nutrition Board of the National Research Council. The Board gives the following explanation of the changes (1):

TABLE 33

Suggested daily allowances of riboflavin

(Food and Nutrition Board, National Research Council, 1948)

Subjects	Calories	Milligrams of Riboflavin		
		1941	1945	1948
Man				
Sedentary	2400	2.7	1.6	1.8
Moderately active	3000	3.3	2.0	1.8
Very active	4500	2.2	2.6	1.8
Woman				
Sedentary	2000	2.2	1.5	1.5
Moderately active	2400	2.7	1.6	1.5
Very active	3000	1.8	2.0	1.5
Last half pregnancy	2400	2.5	2.5	2.5
During lactation	3000	3.0	3.0	3.0
Children under 12 yrs.				
Under 1 yr	110/2.2 lbs.	0.6	0.6	0.6
1–3 yrs	1200	0.9	0.9	0.9
4–6 yrs	1600	1.2	1.2	1.2
7–9 yrs	2000	1.5	1.5	1.5
10–12 yrs	2500	1.8	1.8	1.8
Boys and Girls over 12 yrs.				
Boys				
13–15 yrs	3200	2.4	2.0	2.0
16–20 yrs	3800	3.0	2.5	2.5
Girls				
13–15 yrs	2600	2.0	2.0	2.0
16–20 yrs	2400	1.8	1.8	1.8

> "At the time of formulating the original table of allowances (1941) the results of only two experiments were available for determining the riboflavin requirement, one by Sebrell and Butler (2) and the other by Strong, Feeney, Moore and Parsons (3). In both studies it was found that with an intake of 2 mg. the excretion did not equal that obtained from the control diet, but with an intake of 5 mg. urinary excretion in-

creased sharply. From these results a requirement of 3 mg. was deduced. In appraising the data the committee concluded that somewhat less than this amount might be satisfactory. Another consideration was the relationship which had been shown in experimental animals between thiamine and riboflavin requirements, a ratio of 2 to 3. Adding 50 per cent to the original thiamine allowance of 1.8 mg. for the moderately active man receiving 3000 calories gave an allowance of 2.7 mg., the amount recommended.

"Newer evidence indicates that the former riboflavin allowances were unnecessarily generous, especially at the levels of higher calorie intake. Several groups of workers (4) have shown that intakes of .3 to .35 mg. for each 1000 calories for periods of several months did not result in evidence of deficiency other than reduced urinary excretion. Williams and coworkers (4) found that 0.5 mg. approximated the minimum at which excretions indicated fairly satisfactory stores and that 0.8 mg. is probably in excess of the need. Several workers have shown that 0.6 mg. for each 1000 calories is adequate for maintaining good body stores as indicated by urinary excretions. The newly recommended allowances (1948) for adults and older boys represent reductions of the earlier ones (1941) but not for pregnancy and lactation, for which it was believed desirable to maintain high standards. The new recommendations for riboflavin were influenced also by the finding with growing animals that the riboflavin is related to the thiamine requirement in the approximate proportion of 3 to 2. Because the requirement for thiamine rests on somewhat firmer ground than that for riboflavin, the recommended riboflavin allowance has been kept roughly proportionate to that recommended for thiamine. Furthermore, evidence from experiments by Sherman and associates (5) suggested that short term observations are particularly unreliable in judging requirements for riboflavin. Therefore it seems desirable, until evidence from longer periods of controlled observations on man has been presented, to strive for liberal allowance of this vitamin."[2]

The views of Sherman referred to above were stated by him as follows:

> "In controlled experiments extending through two generations of laboratory animals it has appeared that riboflavin ranked with calcium and vitamin A as the major factors in the improvement of nutritional well being which resulted from an increase in the proportion of milk in an already adequate diet, and that in the experiments thus far completed in which riboflavin was the sole significant variable, excessive increments of intake resulted in successively increased benefits in well being, up to levels more than twice that of minimal adequacy."

Jeans (6) in discussing the recommended allowances states:

> "Evidence for the riboflavin requirement for human beings is meager. The amounts recommended by the Board were influenced by the recorded human experiments as well as by observations with animals. The animal experiments indicated that the riboflavin requirement is related to that for thiamine. The human experiments were made with adults. For other age categories the relationship to thiamine was the chief guide. Obviously more experimental data are needed."

Riboflavin Excretion

There are three paths of exit for riboflavin from the body: In the urine, in the feces, and in perspiration. The last, according to Tennent and Silber (7) is negligible, not exceeding 10 γ per hour.

Fecal excretion appears to be determined largely by the extent of intestinal synthesis which in turn is affected by the character of the diet bu is largely independent of the diet content of riboflavin. Denko et al (8 found the fecal excretion in seven young men always greater than thei urinary excretion and on a low intake (0.36 mg. per day) three times the intake.

Hathaway and Lobb (9) have emphasized the effect of character of the diet. In 3 adult women on a mixed natural diet supplying 1.33 mg. of riboflavin per day in contrast to a synthetic diet supplying the same amount of riboflavin, the fecal excretion on the natural diet was 84 per cent of the intake while on the synthetic diet it was only 28 per cent of the intake. They attribute this difference to increased intestinal synthesis on the natural diet. Others (10) have reported the fecal excretion to be independent of the intake.

Fecal excretions, then, are of little value in estimating riboflavin requirement but they have been widely studied to determine the relation of dietary ingredients to their production. In Section I was cited the work of Mannering et al (11) who reported that dextrin, cornstarch, and lactose favor the development of the bacteria that synthesize riboflavin more than does sucrose or an increase of fat in the diet. If this bacterially synthesized riboflavin is available for absorption, it could influence the amount of riboflavin required in the diet.

Czaczkes and Guggenheim (12) made a rather extensive study of the relation of diet ingredients to bacterial synthesis by the rat. They used five experimental diets, viz. one called *normal* which consisted of 15 per cent casein, 71 per cent rice flour, 10 per cent olive oil and four per cent salt mixture plus vitamin supplements but no riboflavin. By varying the proportions of casein, rice flour and oil four other diets were prepared: A *high protein* diet (34 per cent of the calories from protein); a *low protein* diet (11 per cent of the calories from protein); a *high fat* diet (40 per cent of the calories from fat); a *low fat* diet (2 per cent of the calories from fat). Each of these five diets were subdivided to provide different amounts of riboflavin supplement. The effects pertaining to the matter of bacterial synthesis were as follows:

Forty-eight hour collections of feces from rats on the normal diet with no riboflavin supplement averaged 24 γ and about the same on the low protein diet. On the high protein diet the fecal excretion was only 11 γ and on the high fat diet only 9 γ. On the low fat diet they excreted 35 γ. The reduced excretion on the high protein and high fat diets and the increase on the low fat diet correlated with respectively low and high levels of tissue storage and with urinary excretions. There was no such correlation on the low protein diet. They consider this result supports the view that on a low protein diet the ability to store riboflavin in the tissues is reduced and urinary excretion is increased though, since they do not report the total daily urinary excretions, their figures do not fully support this conclusion.

The figures did, however, supply evidence to support the hypothesis that the differences in fecal excretions were due to different rates of intestinal synthesis by bacteria. They counted the viable cells in the feces on the different diets, incubated bacteria from the feces, and measured their extracellular output of riboflavin. Feces from rats on the normal diet with no riboflavin supplement gave a count of 318 million viable cells per gram, from the low protein diet 450 million cells. On the high protein, and the high fat diets the numbers were one third as many. These figures indicate that the fecal excretion of riboflavin is proportional to the number of viable bacteria in the feces and presumably to the amount of riboflavin they synthesize. This was supported by the check on amounts produced by the fecal bacteria.

The effect of nitrogen balance on fecal and urinary excretion in human subjects has been reported by Oldham et al (13). They reported that the urinary excretions varied inversely with the nitrogen balances; 40–60 per cent of the intake when the balance was positive, 7 per cent when it was negative; and fecal excretions not affected.

It appears to be established that fecal riboflavin is mainly bacterially synthesized and that certain ingredients in diet affect the activity of the synthesizing bacteria. To what extent the riboflavin formed by the intestinal bacteria is released in the gut and absorbed therefrom is not so well established. Najjar and Barrett (14) state that probably most, if not all, of the riboflavin so synthesized is held in the bodies of the bacteria and excreted in the feces, and liberation occurs only after bacterial disintegration.

Urinary Excretion and Riboflavin Requirement

As already noted, the Food and Nutrition Board's 1941 allowances were based primarily on two reports (2, 3). The Strong et al (3) report gave the daily urinary excretion on unrestricted diets as 500–800 γ in 24 hours which rapidly decreased to 50–150 γ on an intake of 1–2 mg. of riboflavin per day, amounts they considered perhaps insufficient to supply satisfactorily the daily requirement. But they found that extra amounts (2–5 mg.) were promptly excreted.

Some other studies on excretion on unrestricted diets were made in the period before publication of the 1941 allowances. Emmerie (15) in 1936–37 reported that the daily elimination in the urine of males is from 819–1250 γ per day, but that if the intake is increased there is a corresponding increase in the amount eliminated. On the basis of Emmerie's results, Hogan

(16) suggested that a man should receive from 2–3 mg. of riboflavin pe day.

The second study referred to by the Food and Nutrition Board was by Sebrell et al (2) and was the first detailed, long-time study of the relation of urinary excretion to known amounts of intake. They found that women on an institutional diet excreted an average of 357 γ of riboflavin per day; that on a measured intake of 1.56–2.05 mg. daily the excretion ranged from 140–186 γ per day while on an intake of 2.54–3.68 mg. per day the excretion was from 793–1265 γ, an amount higher than that excreted by the women on the institutional diet and within the "normal" range for men reported by Emmerie (15) and by Ferrebee (17). From their data they express the conclusion that the adult daily requirement should be approximately 3 mg.

In the period between 1941 and 1948 the matter of urinary excretion of riboflavin and its relation to human needs has received rather extended investigation. The results of a typical study of this sort by Davis et al (10) are shown in Table 34. This table gives the findings with one woman out of twelve women used in the test. Some of the conclusions drawn from the data by the investigators illustrate the questions at issue in the urinary secretion studies. For example they claim, contrary to the findings of Feder et al (18) and in agreement with Hathaway and Lobb (9), that the fasting rate of excretion was more constant per unit of time than per unit of volume. They found the fecal excretion to be independent of the riboflavin intake, and during the low riboflavin intake the fecal output was in excess of the intake. This agrees with Denko et al (8). They noted no holdover following a large dose as shown in the return to average urinary output in period V following period IV.

As in the case of Sebrell et al (2) and others, the failure of the urinary excretions to account for a considerable percentage of the intake supports the contention that part of the intake is destroyed in the tissues.

In their series of tests no clinical evidence of ariboflavinosis appeared in subjects who remained on the low intakes (0.27–0.29 mg.) for over a hundred days, which indicates no correlation of clinical response and urinary excretions, in agreement with Hagedorn et al (19)and Keys et al (4).

Their conversion of these data into an estimate of requirement is also of interest in illustrating present tendencies. They concluded that a daily intake of 0.5 mg./1000 calories satisfies the needs of normal young women since in period III forty per cent of the additional riboflavin supplied was excreted in the urine whereas only 11 per cent of the increment in period II was eliminated; furthermore small increases in the fasting excretion and the test dose response occurred in period III coincident with the increase in riboflavin from 1.01 to 1.24 mg. daily. In this conclusion they concur with Williams et al (4).

Some other findings are of interest in the matter of evaluating urinary excretions. Johnson et al (20) consider fasting values superior in significance to random or to oral load responses.

Jeans (6) has noted that there are little data on children's requirements. Oldham et al (21) have reported one study with 5 year old boys. They concluded that constant urinary excretion of 117–105 γ on an intake of 0.53 mg./1000 calories indicated adequate allowance; also that a one hour fasting excretion of 9 γ was satisfactory evidence of adequacy.

TABLE 34

A typical study of urinary excretion of riboflavin

(After Davis et al. (10) 1946)

Test periods	Number of days on diet	B2 intake		24-hour urinary excretion	Intake in urine	Fasting urine excretions	Intake in urine	Test dose returned	24-hour fecal excretion
		Total	Per 1000 calories						
		mg.	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*	*per cent*	
Preliminary	7					0.017		20	
I	106	0.54	0.25	0.08	14.7	0.008	1.47	4	0.5–0.65
II	45	1.01	0.47	0.14	13.9	0.012	1.18		0.5–0.65
III	41	1.24	0.65	0.23	18.5	0.015	1.20	13	0.5–0.65
IV	14	7.22		4.48	62.0				Not measured
V	20	1.26	0.68	0.23+	18.2	0.014	1.11	10	0.5–0.65

Comment: The diet in the preliminary period was of self selected food stuffs (excluding pork and liver). In the other periods a selected diet of known riboflavin content was fed. This diet supplied 49–62 grams of protein; 9.7 to 14.3 mg. of niacin; 2.3–4.7 mg. of pantothenic acid and supplements of iron, calcium vitamin A and ascorbic acid throughout the 5 periods. The thiamine intake was controlled and increased from 0.14 mg./1000 calories in period I to 0.54 mg./1000 calories in the final period.

Throughout the study 72-hour collections of urine and feces were made each week (except for the first week) after each dietary change.

The excretions in one-hour fasting urines and the per cent return in four-hour urine after the test dose of 0.02 mg./kilo of body weight were determined the last week of each period.

Roderick et al (22) have studied the problem of lactation requirement. On an intake of 3 mg. of riboflavin daily the secretion into milk was 9 per cent of the intake and the urinary excretion 47 per cent of the intake; leaving 44 per cent unaccounted for.

This review does not attempt to cover all the studies of urinary excretion and its value as a basis for estimating requirement but it should be evident that with failure of correlation with clinical manifestations of deficiency, with uncertainty as to the utilization of intestinally synthesized riboflavin, with lack of long term studies to determine effect on longevity the estimates of the Food and Nutrition Board are far from satisfactory, but may well serve for trial until they are shown definitely in need of further change.

Riboflavin Storage

As in the case of thiamine, there is little storage of riboflavin and hence necessity for daily renewal of supply in the diet. As shown in Table 35 Taylor et al (23) have reported the distribution found in human tissues and that the major storage tissues are the liver and kidney.

TABLE 35

Distribution of riboflavin in human tissues

(After Taylor et al. (23) 1942)

Tissues	Micrograms of riboflavin per gram of fresh tissue
Heart	7.81–19
Kidney (renal cortex)	13–25
Liver	16–18
Skeletal muscle	1.8–2.9
Smooth muscle	2.3
Brain	2.1–2.8
Lung	1.6–1.9
Spleen	3–7.2
Adrenals (whole)	6.8 –11.0
Ovary	4.3
Testes (sem. tubules)	1.7–2.3
Skin	0.85–1.5

Animal Requirements for Riboflavin

Cattle: Since riboflavin, like thiamine, is synthesized in the rumen of cattle they are to a large extent independent of dietary intake after they have reached the age of two months and rumen synthesis is established. However, deficiency in other ingredients of the diet such as protein which can influence the synthetic activity of the bacteria can create riboflavin deficiency. For young calves the mother's milk should be adequate supply up to weaning, it averages 1.7 mg. per quart. Incidentally it is important to human milk consumers to know that exposure of their milk bottle to the sun can destroy much of its riboflavin content.

Sheep: These animals, like cattle have rumen synthesis and secretion in their milk, hence they are largely independent of dietary intake.

Swine: For growing pigs riboflavin supplement is very important. Lack of adequate intake may result in stiffened limbs, a sebaceous exudate over back and sides, secretions around the eyes, cataract, nerve degeneration, and yellow livers. Requirement per 100 lbs. of live weight are put at 3.8 mg. for growing pigs. Less is known of the requirements for mature animals.

Poultry: Poultry also require definite amounts of riboflavin in the feed. In young chicks deficiency results in diarrhea, retarded growth, and leg paralysis. The paralysis involves the legs and feet and occurs in two stages, a preliminary stage which is curable and an acute stage that is incurable. Curled toe paralysis is a typical riboflavin deficiency effect.

In breeding birds riboflavin deficiency results in poor hatchability of the eggs, and the requirements for hatchability are considerably higher than for normal egg production and maintenance of health. An average hen's egg contains 0.16 mg. of riboflavin. The recommended dietary intake for starting chickens is 1.6 mg. per pound of feed; 1.3 mg. per pound for laying and breeding hens. For turkey poults the recommendation is 2 mg. per pound and 1.8 mg. per pound for turkey breeders.

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CHAPTER IX. NIACIN (VITAMIN P-P)

SECTION I. FUNCTIONS OF NIACIN

Niacin is a specific for the disease known as pellagra but as a component of the coenzymes I and II it is also involved in oxidation systems in the body and in the metabolism of carbohydrates in particular.

Niacin (nicotinic acid) was first described by Huber (1) in 1867. It can be produced from nicotine but has none of its toxic properties. Though it was isolated from rice and yeast by Funk (2) in 1911–13, and from rice by Suzuki et al (3) in 1912 these investigators at the time were seeking a cure for beri-beri and the nicotinic acid had no effect on that disease but Funk (43) noted that it enhanced action where added to the anti-beri-beri fraction. That it was a specific remedy for black tongue in dogs (the analogue of human pellagra) was first demonstrated by Elvehjem et al (4) in 1937.

Pellagra

The following facts are of interest in the campaign against the disease known as pellagra, endemic in certain parts of the United States and the most dangerous avitaminosis known in this country. It was first studied by Casal in 1725 in the province of Asturia in Spain who called it "mal de rosa" and one of its manifestations is still called "Casal's necklace". Later the disease became prevalent in Milan and it was Frapoli who gave it its name of "pelle agra" or rough skin, later contracted to pellagra. The first case was described in American literature by Gray in 1864. Isolated cases became more numerous as time went on, and Searcy experienced a serious epidemic of the disease in a hospital for the insane in Alabama in 1905. Soon afterward it was discovered that the disease was endemic in Alabama, Mississippi, Louisiana, Texas, Georgia, the Carolinas, Kentucky and Tennessee and as recently as 1941 there were 1868 deaths in the United States due to pellagra.

The suggestion that it was a nutritive deficiency disease was first made by Funk in 1914. Funk's viewpoint was thoroughly tested and confirmed by Goldberger and coworkers of the U. S. Public Health Service and to them is due credit for pinning the disease to deficiency of some factor in certain foods and identifying a group of foods that supplied the factor and could be used to prevent the deficiency.

Goldberger's work was handicapped at first by lack of a suitable experimental animal for use in its study. This problem was solved when it was found that a disease known as "black tongue" in dogs was a true analogue of human pellagra and responsive to the same curative and preventive measures. This fact was what made possible Elvehjem et al. demonstration that niacin was the preventive vitamin.

Characteristics of Pellagra

Classic pellagra is a syndrome in which the skin, the alimentary tract and the central nervous system are all affected. Spies, Vilter and Ashe (5) have given a comprehensive discusssion of its diagnosis and also recognized that the disease, as it occurs in patients, is rarely a manifestation of a single dietary deficiency; that it is commonly accompanied by symptoms due to deficiency of vitamins B_1 and B_2.

The following description is taken from the discussion of Spies et al. (5):

> "Symptoms arising from involvement of the alimentary tract usually are the first to be noted. Nonspecific prodromal symptoms of anorexia, abdominal pain, and burning of the tongue gradually progress to intense glossitis, stomatitis, gingivitis, pharyngitis, gastritis and enteritis. The lips may assume an erythematous and cracked appearance, while the tongue becomes fiery red, swollen, and smooth except where it is indented by the pressure of the teeth. The gums become red and ulcerated, and the pharynx may take on the same appearance. Gastroscopic studies have revealed fiery red ulcerated lesions of the mucous membrane, similar in appearance to those observed in the mouth. Achylia has been reported to occur in 60 percent of the cases. The enteritis and proctitis result in a foul, watery diarrhea, severe abdominal pain, and abdominal distention.
>
> The characteristic dermatitis is bilaterally symmetrical and appears most often on the dorsa of the hands, the elbows, knees, neck, and axillae, and in the perineal region. The affected skin is roughened (pelle agra), erythematous, desquamating, cracked, and sharply demarcated from the adjacent, healthy skin. The lesions are progressive and constantly changing, with older lesions inclined to be more highly pigmented.
>
> The mucous membranes of the urogenital tract often are affected and may become the site of secondary infections. Severe vaginitis, urethritis, and endocervicitis are common implications. Vincent's organisms are found in abundance wherever the mucous membranes are affected by pellagra.
>
> Mental symptoms, which are common in classic pellagra, often are of a paranoid type in the later stages, and may be accompanied by visual hallucinations, delusions of persecution, depression and recessiveness. Patients at first become excited, irritable, and eventually dangerously maniacal. Delirium may occur.
>
> The administration of niacin or niacinamide in adequate dosage frequently produces dramatic improvement within twenty-four to forty-eight hours. Insight returns, the patient becomes quiet and cooperative, and all evidences of mental disease may disappear. If mental symptoms have gone untreated for weeks or months, however, a longer period of time under adequate niacin therapy is necessary for improvement and complete recovery may never be attained. When degenerative changes have occurred these obviously cannot be influenced by niacin therapy.
>
> Deficiencies of thiamine and riboflavin are frequently associated with pellagra, and it may be necessary to administer these vitamins also, in order to obtain improvement of the lesions for which they are specific. A liberal and well balanced diet is essential for continued improvement (6)."

The relations of nicotinic acid and its amide to pellagra is stated as follows by the Council on Pharmacy and Chemistry of the American Medical Association (7):

> "Nicotinic acid ($C_6H_5O_2N$) and nicotinamide ($C_6H_6O\ N_2$) are of fundamental importance in the treatment of pellagra. The terms niacin and niacinamide are now officially recognized as synonyms for these chemical names. . . .Sufficient evidence has now been accumulated to demonstrate the usefulness of these drugs. Administration of relatively large doses of nicotinic acid produces a marked flushing of the face and neck. There is an unpleasant sensation but the reaction is transient and apparently harmless. The effect is not observed following the administration of nicotinamide. For parenteral use nicotinamide is the drug of choice. *Allowable Claims:* Nicotinic acid and nicotinamide are recognized as specifics only in the treatment of pellagra. Their administration in appropriate doses leads to the disappearance of all alimentary, dermal, and other lesions, characteristic of the disease, to a return to normal of the porphyrin and porphyrin like pigments of the urine, and to a profound improvement in the mental symptoms when the latter are the result of an inadequate intake of nicotinic acid and nicotinamide. These compounds are without influence upon the polyneuritis or cheilosis so frequently observed in pellagrous patients. In such cases it may be necessary to insure the presence in the diet of foods rich in vitamins B_1 and B_2, or to administer thiamine hydrochloride, riboflavin or both."

Relation of Corn (Maize) to Pellagra

As early as 1735 Casal argued that pellagra was due to some toxic or infectious factor present in maize (corn). In 1940 Aykroyd and Swaninathan (8) reported that certain groups in India, eating a staple rice diet providing only 5 mg. of niacin daily, rarely developed pellagra. In another group on the staple corn diet of Moldavia pellagra was endemic though this diet supplied as high as 15 mg. of niacin daily.

A similar situation in this country was reported by Dann (9). He noted that in Wayne County, North Carolina, where the daily diet provided only 7.5 mg. of niacin but was low in corn, there was little pellagra whereas Goldberger and Wheeler's (10) experimental diet containing an equal quantity of niacin produced pellagra when corn was added.

The final explanation offered was that the effect of the corn was not due to its low content of niacin but to its deficiency in the amino acid tryptophan. In 1945 Krehl et al (11) noted that when 2 parts by weight of corn meal or corn grits was added to 3 parts by weight of a low protein diet the result was a reduction in the growth of rats; that this effect could be prevented by addition of 1 mg. of niacin per 100 grams of ration or by raising the casein content from 15 to 20 per cent. This latter finding suggested an amino acid deficiency. To test this they (12) tried the effect of adding tryptophan or lysine instead of niacin. They got no effect with lysine, but 0.05 per cent of l-tyrptophan was as effective as the addition of the niacin. The fact that the addition of polished rice instead of corn produced no such growth retardation, though white rice contained less niacin than corn but more of tryptophan, confirmed the significance of tryptophan (See Table 36).

At the time the suggestion was made that tryptophan was an essential metabolite for the activity of the bacteria which produce niacin in the intestine. In tryptophan deficiency there would be reduction in the intestinal synthesis of niacin and hence an increased requirement for niacin in the diet (13, 14).

TABLE 36

Comparison of rice and corn as sources of tryptophan and niacin

A. Tryptophan comparison

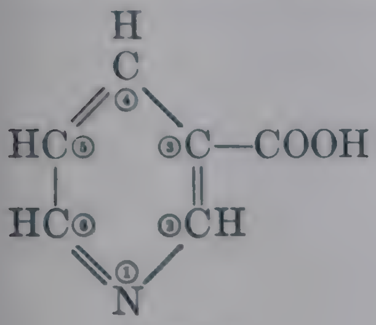
Foodstuffs	Tryptophan
	per cent
Brown rice	0.074
White rice	0.066
White corn	0.047
Whole wheat	0.080
Casein	0.950

B. Comparison of niacin content

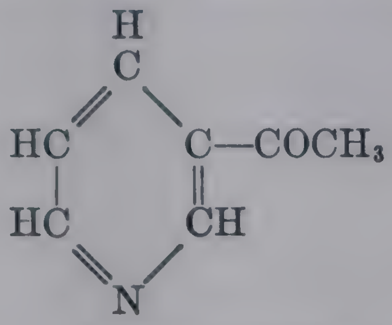
Rice products	Niacin per lb.	Corn products	Niacin per lb.
	mg.		*mg.*
Brown rice	20.7	Yellow corn meal	9.3
White rice	6.3	White corn meal	7.9
Converted rice	17.2	Degerm. yellow cornmeal	4.1
Crisp rice cereal	28.8	Degerm. white cornmeal	4.3
Puffed rice	24.0	Enriched yellow cornmeal	16.0
Rice flakes	19.2	Enriched white cornmeal	16.0
		Crisp corn cereal	5.1
		Corn flakes	8.0

Another viewpoint that gained strength with increasing research, was that tryptophan is an actual precursor in niacin synthesis and not simply a stimulant to bacterial activity in niacin synthesis. Both Singal et al (15) and Rosen et al (16) have submitted evidence to indicate that niacin is actually synthesized from tryptophan, that administration of tryptophan results in an increase in the urinary excretion of niacin *but not of the fecal excretion of niacin*. The present viewpoint is that this conversion takes place in the tissues and not as a result of action of intestinal microorganisms. [Incidentally another vitamin, pyridoxine, appears involved in this synthesis of niacin (17)]. This viewpoint has been further supported by Bell et al (18) who also suggest that amino acids other than tryptophan may be involved in niacin synthesis.

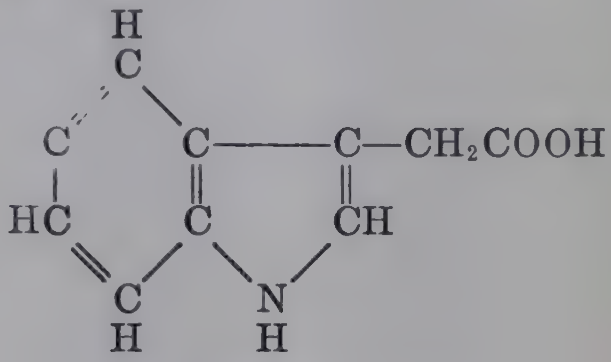
But there is also new evidence that suggests that the old view of a toxic factor in corn may not be wholly wrong. In 1938 Woolley (19) noted the pellagrenic action of certain pyridines on dogs; that 3-acetyl pyridine was lethal to niacin-deficient dogs but not to normal dogs. In 1945 he reported that pellagra symptoms could be produced in mice by feeding 3-acetyl pyridine and in 1946 that its action could be counteracted by either tryptophan or niacin. In the same year Kodicek, Carpenter and Harris (20) showed that the feeding of indole-3-acetic acid to rats produced pellagra symptoms similar to that resulting from feeding corn. Woolley (21) has concentrated a factor from corn which in as small amount as 1 mg. per 100 grams of ration retarded mouse growth. He has not to date reported on the chemical nature of his compound but it may well be the indole-3-acetic acid of Kodicek



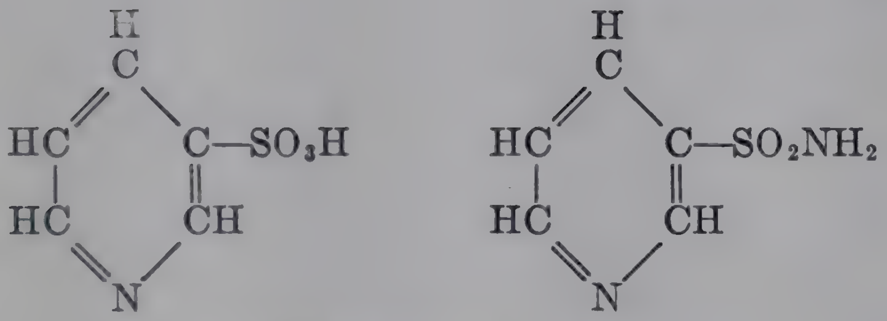
Niacin



3-acetyl pyridine



Indole-3-acetic acid



Pyridines-3-sulfonic acid or amide

Fig. 15. Anti-Niacin Compounds and Their Relation to Niacin

et al. We have here a situation analogous to thiamine and the existence of anti-vitamin compounds which counteract the niacin. For the relation of those reported to niacin see figure 15.

The Role of Niacinamide as the Prosthetic Group in Coenzymes I and II

Buchner first proved that the alcoholic fermentation produced by yeast was through the action of a protein containing enzyme to which he gave the name zymase. Harden (25) showed that if one added to a phosphate-dextrose solution some of Buchner's zymase the fermentation was greatly quickened if some mammalian blood serum was added. Von Euler later obtained a similar enhanced fermentation by adding boiled and filtered autolysed yeast. The substance in the blood serum and the yeast was named cozymase; unlike the enzyme it was heat stable. It was not, however until nearly thirty years later that the chemical structure of cozymase was determined. In 1934–5 Warburg and Christian (26) demonstrated that niacinamide was a constituent of a cozymase or coenzyme found in horse blood and that it functioned in hydrogen transport. This coenzyme is known today as coenzyme II. The coenzyme found in yeast, now known as coenzyme I was also shown by Euler, Albers, and Schlenck (27) to contain niacinamide, to function in hydrogen transport, but to contain one less phosphoric acid molecule than Coenzyme II. Both coenzymes function in respiratory oxidation systems as carriers of hydrogen.

Schopfer (28) has pictured their position in a typical hydrogen transport system by which hydrogen is removed from the substrate and ultimately combined with oxygen to form water and release energy as follows:

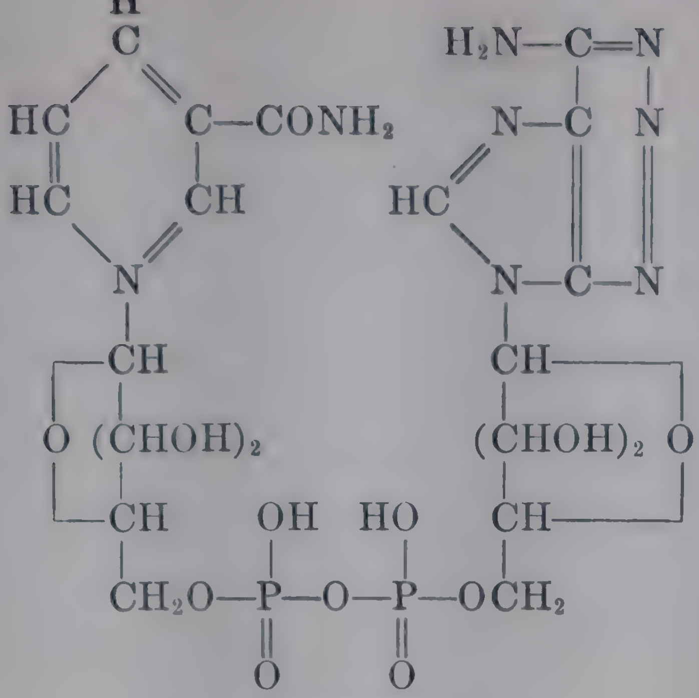
$$H_2 \text{ substrate} \rightarrow \text{coenzymes} \rightarrow \text{yellow enzyme} \rightarrow \text{cytochrome} \rightarrow 0$$

In 1936 Lwoff and Lwoff (29) pointed out the similarity in properties of coenzymes I and II to what had been designated the "V" factor necessary for the growth of certain influenza bacilli. And later that they were identical. This discovery made it possible to devise a microorganism test for their presence in tissues.

It is now certain that they function in the tissues in the metabolism of carbohydrate and hence are essential to growth and activity. The question then arose: how does ingestion of niacin or niacinamide produce the coenzymes? Also, is it the action of the coenzymes in the tissues that prevents and cures pellagra?

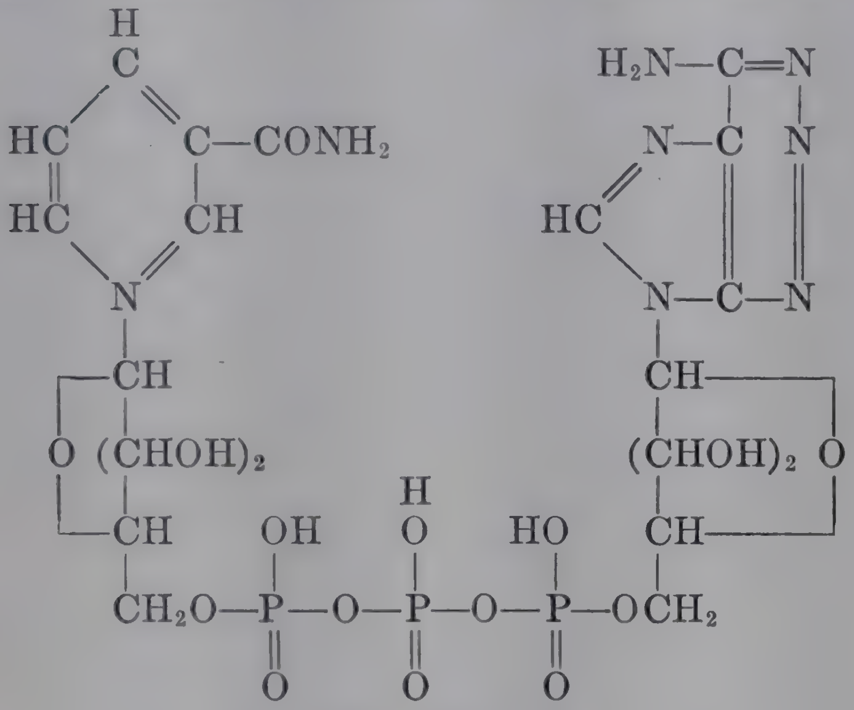
Schlenck (30) has reviewed the research that supplied answers to these questions. There is not space to give credit to all who worked on these problems but the following are some of the significant contributions.

Coenzymes are synthesized in the tissues from ingested niacin or niacinamide. These vitamins diffuse from the plasma into the erythrocytes, and in the blood the synthesis occurs in the erythrocytes. Formation also takes



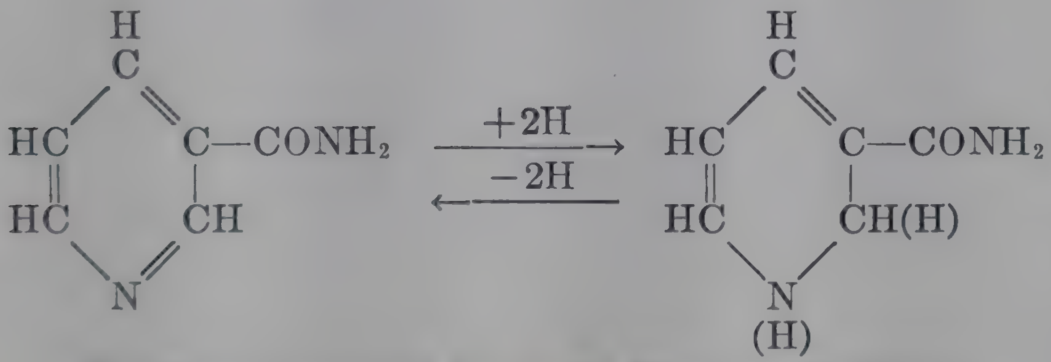
Coenzyme I

(After Euler Hv. Schlenck F. & Gunther G. Z. Physiol. Chem. 233: 120 (1935).)



Coenzyme II

(After Warburg O., Christian W. & Griese W. Biochem Z., 282:157 (1935) & 287:291 (1936).



Hydrogen transport due to the Niacinamide Group

Fig. 16

place in tissue cells. Niacin deficiency decreases the coenzyme content of liver and muscle which is rapidly restored to normal by feeding niacin. (31). But it was also shown that feeding niacin in excess over requirement does not as a rule increase the coenzyme content of the tissues above the normal level (32, 33). An exception was the blood level of coenzyme I which could be increased by large amounts of niacin, the increase being proportional to the amounts fed (34). However the decrease in coenzyme content of the liver and muscles of dogs with black tongue cannot, according to Dann and Handler (35), be the immediate cause of death. They suggest that dehydration or the toxic reactions of inflammation after invasion of the alimentary mucous membranes may be such cause. It is also known that feeding coenzymes instead of niacin or niacinamide does not cure pellagra(36). There has been some question as to whether niacin and niacinamide are equally available for formation of coenzyme. Axelrod et al (48) reported niacinamide effective for coenzyme synthesis; Handler and Kohn (49) that it was only one third as potent as niacin. Leifer et al (50) have later reported a study of the behavior of the compounds in human blood and that while human red cells did utilize the niacin for coenzyme formation the niacinam-

TABLE 37

Coenzymes I & II

(After Schlenck (30) 1942)

A. Properties of coenzymes I and II

Properties	Coenzyme I	Coenzyme II
Empirical formula.......	$C_{21}H_{28}O_{14}N_7P_2$	$C_{21}H_{29}O_{17}N_7P_3$
Molecular weight........	663	743
Structural units........	1 mol niacinamide 1 mol adenine 2 mols pentose 2 mols phosphoric acid	1 mol niacinamide 1 mol adenine 2 mols pentose 3 mols phosphoric acid

B. Distribution of coenzymes I and II

Material examined	Micrograms per gram of fresh material	
	Coenzyme I	Coenzyme II
Bottom yeast..................................	>500	<10
Top yeast.....................................	>500	5–10
Erythrocytes (horse)..........................	100	>12
Liver (rat)...................................	>200	30
Muscle (rat)..................................	200	50
Kidney (rat)..................................	160	40

ide was not so utilized. However mouse erythrocytes in vitro did use both to a similar degree. They point out that there is a species difference in this utilization to be taken into consideration.

From the nutrition viewpoint, animals, some bacteria, and plants require niacin or niacinamide to enable them to carry on their normal metabolism which results in growth, weight maintenance and activity. In this case the conversion of the vitamins to the coenzymes and their participation in respiratory oxidation explains how they function. The explanation of how niacin and niacinamide actually prevent and cure pellagra is still unexplained and waits further study of the metabolism of these enzymes in the organisms. Progress has been made in the metabolism of niacin and niacinamide and is reviewed in Section II.

Pharmacological Functions of Niacin

It has been noted that niacin produces a vasodilation or flushing; an effect not produced by niacinamide. Use of this vasodilation effect has been made in the treatment of angina pectoris (37, 38). Johnson (39) has reported it to be valuable in the treatment of Vincent's infection and Sydenstricker and Cleckley (40) that it is of value in the treatment of psychosis in patients who gave no signs of pellagra. It is claimed that it was effective in promoting gastro-intestinal motility in dogs with black tongue and in human beings with pellagra, an effect not produced by dosage of either thiamine or riboflavin (41). Also it promoted the healing of fractures (42).

Its main value however is as a specific for pellagra and as a metabolic factor in normal nutrition.

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SECTION II. FORMS AND CHEMISTRY OF NIACIN

Niacin and niacinamide are derivatives of pyridine. Of these derivatives only three, niacin, niacinamide, and coramine have been found suitable for the treatment of human pellagra. In the laboratory it is possible to convert nicotine into niacin but no such transformation occurs in the body and niacin has none of the toxicity of nicotine. It was to avoid confusion with nicotine that the terms nicotinic acid and nicotinic acid amide were changed to niacin and niacinamide (See figure 17).

In Table 38 are listed derivatives of nicotinic acid that have been found active and inactive according to Elevehjem (1). It is evident from a study of figure 17 that the carboxyl group or the amide in position 3 of the pyridine ring is essential for activity.

TABLE 38

Some active and inactive pyridine derivatives

(After Elvehjem (1) 1940)

Active derivatives	Inactive derivatives
Niacin	Picolinic acid
Niacinamide	Iso-nicotinic acid
Ethyl nicotinate	Nipicotic acid
Nicotinic acid N-methyl amide	6-methyl nicotinic acid
Nicotinic acid N-diethyl amide	Trigonelline
Beta picoline	1-methyl nicotinic acid amide HCl
Nicotinuric acid	Quinolinic acid
	Beta amino pyridine

As shown in figure 16 it is the niacinamide that provides the prosthetic group in Coenzymes I and II.

Properties of Niacin and Niacinamide

Niacin is a white odorless crystalline powder with a slightly acid taste. One gram is soluble in 60 cc. of water or in 80 cc. of alcohol at 25° C. It is freely soluble in boiling water, boiling alcohol, and in aqueous solutions of alkali hydroxides and carbonates, but almost insoluble in ether. The pH of a one per cent aqueous solution is approximately 3.

In the dry state is it quite stable; melting point 234–237° C. Niacinamide is also a white odorless, crystalline powder with a slightly bitter taste. One gram is soluble in 1 cc. of water or 1.5 cc. of alcohol or 10 cc. of glycerin. Solublility in benzene or ether is slight.

The pH of a 1 per cent aqueous solution is approximately 6.

In the dry state it is also quite stable if kept below 50° C. The melting point is 129–131° C.

Both solutions are non-toxic in therapeutic doses.

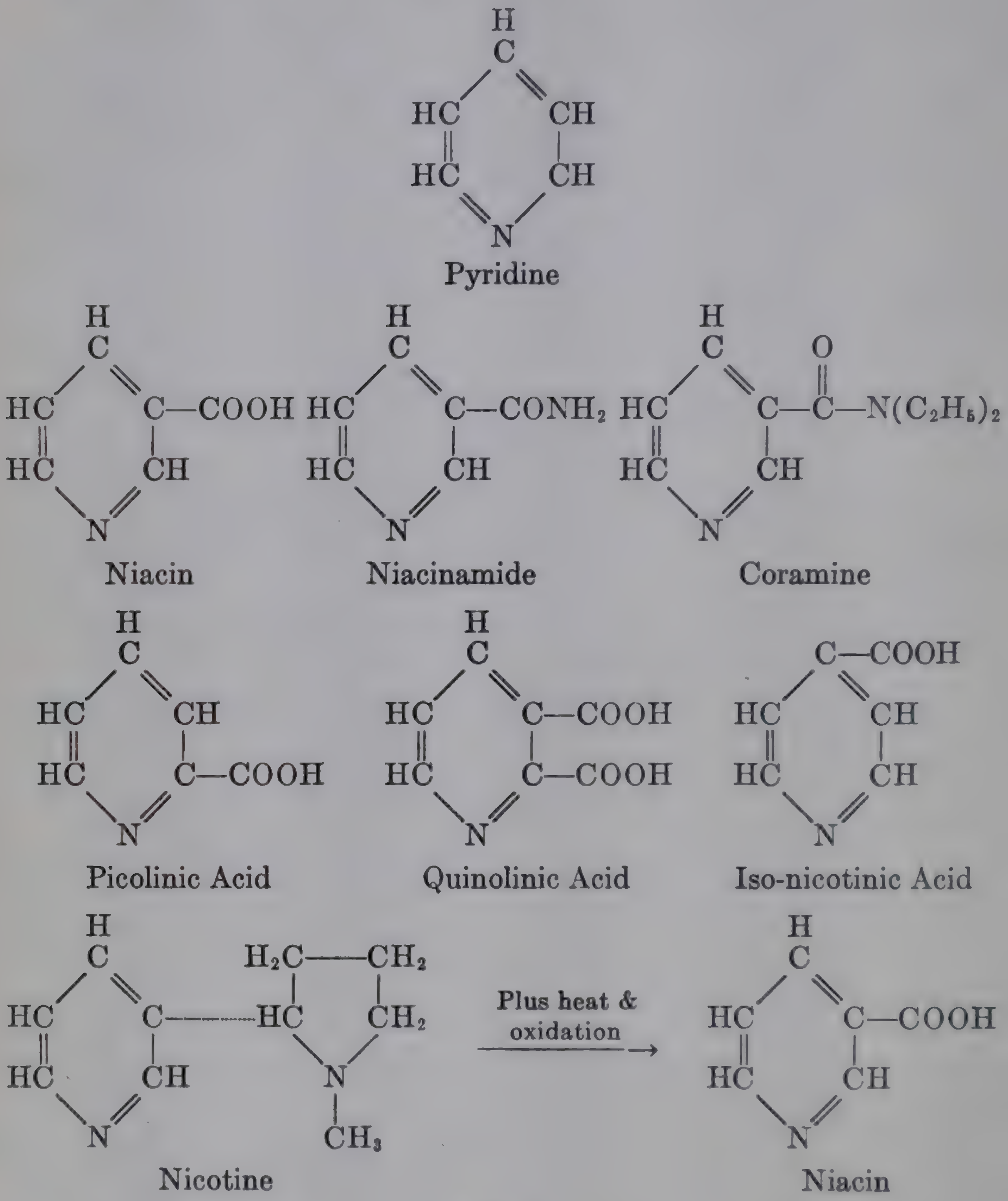
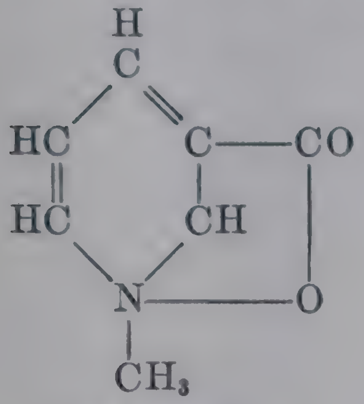
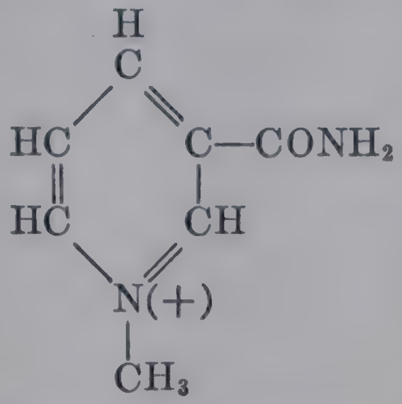


Fig. 17

The Metabolism of Niacin

After the ingestion of niacin, derivatives appear in the urine. These have been identified as free niacin and niacinamide, nicotinuric acid, trigonelline, and N′-methyl- niacinamide or F_2 (See figure 18). Of these the major derivative is F_2, N′-methyl niacinamide. However, much of the ingested niacin is unaccounted for by the sum of these various urinary excretion products. Sarrett et al (2) reported that not more than forty per cent of ingested niacinamide could be traced. Ellinger and Coulson (3) found that four fifths of a dose of N′-methyl niacinamide failed to appear in the urine and must therefore, be metabolized in ways at present unknown. Perspiration accounts for only a small fraction. Cornbleet et al (4) reported excretion of 0.5 mg. in 8 hours; about one quarter free niacin, one quarter free niacinamide, and two thirds N′-methylniacinamide.


Trigonelline


N′-methylniacinamide or F2

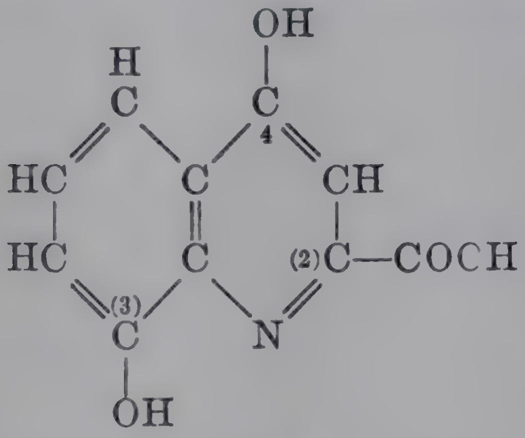

Xanthurenic Acid
4,8-dihydroxy quinoline-2-carboxylic acid

FIG. 18. URINARY EXCRETION FORMS OF NIACIN

Whether or not nicotinuric acid is a urinary excretion derivative is in question. Melnick et al (5) failed to find it, while Perlzweig et al (6) claim it to be present in small quantity. Knox and Grossman (7) have reported finding another derivative in human urine, a more completely oxidized form of N′-methylniacinamide, namely: N′-methyl-6-pyridone-niacinamide.

Until the ingested niacin is fully accounted for, the determination of excretion of N′-methylniacinamide does not form a fully satisfactory basis for estimation of human requirement of niacin or niacinamide. The situation is similar to that in the case of riboflavin. It is hoped that studies reported in part by Beadle and Bonner et al (8) may throw further light on the synthesis of niacin in the body and the intermediates involved in its metabolism.

N′-methylniacinamide

In 1940 Najjar and Wood (9) described a specific reaction in urine which apparently depended upon the store of niacin in the body. When urine was adsorbed on zeolite and subsequently eluted with KCl, treatment of the eluate with NaOH developed a bluish fluorescence. At the time the substance responsible for the fluorescence was not identified chemically, but could be measured quantitatively by the fluorphotometer. It was shown that this substance increased after the ingestion of niacin. Later these investigators reported two of the fluorescent substances and named them F_1 and F_2. Huff et al (10) identified the F_2 as N′-methyl-niacinamide.

The relation of F_2 to niacin metabolism has been further complicated by evidence that tryptophan (now considered a precursor of niacin) gives when fed an increased urinary excretion of F_2 (11, 12, 13, 14, 15).

There are, then, still many gaps in our knowledge of niacin metabolism in the body, and no laboratory diagnosis of satisfactory accuracy is possible today (See Section III).

Niacin Toxicity

While it is true that neither niacin or niacinamide is toxic to human beings in therapeutic doses, they do have a toxic limit. Unna (16) found the oral lethal dose (LD_{50}) of sodium nicotinate for rats and mice was 5.0–7.0 grams per kilo of body weight and 4.0–5.0 grams per kilo of body weight when given subcutaneously. Brazda and Coulson (17) found the LD_{50} of niacin for rats when given subcutaneously was 5.0 grams per kilo of body weight. Niacinamide is three times, pyridine five times and coramine twenty-one times as toxic as niacin.

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Section III. Evaluation of Niacin in Human and Animal Nutrition

Table 39 shows the daily allowances of Niacin recomended by the Food and Nutrition Board of the National Research Council (1). The Board explains its basis for allowances as follows:

> "At the time of formulating the original table of allowances no human experiments on which to base a requirement were on record. The recommended values were based largely on calculations from the requirement of dogs as found by Elvehjem and on the checking of the calculated values against diets known to be adequate for prevention of pellagra. When the allowances were thus calculated for different ages, it was found that they were approximately ten times the thiamine allowances. This observation gave support to the computed values in that the requirement of these two materials might be expected to bear a constant relationship to each other because they, together with riboflavin, play a part in the oxidation-reduction systems of the body, through which the energy of foods is released. At the time of the 1945 revision, evidence as to niacin requirement still remained less adequate than for many other factors. Decision was made to retain the ten to one relationship with thiamine, a procedure which decreases the recommended allowances at levels of higher calorie intakes. It should be pointed out that the niacin intakes of children may be adequate even when less than the recommended allowances, provided the diet contain adequate quantities of milk."

Jeans (2) comments that the maintenance of this 10:1 niacin/thiamine ratio probably allows for maximal need, inasmuch as possible synthesis with a generous supply of milk is considered. Also, the relationship of the niacin need to the amount of tryptophan in the diet needs to be clarified.

If it were possible to estimate needs by relation between excreted niacin and intake it would be possible to establish requirements experimentally but the difficulty here, as in the case of riboflavin, is that not all of the ingested niacin has been yet accounted for in the measurement of the known excretory products.

Blood Level Studies

Studies have been made of the niacin content of the blood in the hope of establishing a normal content. Some reports are available. Oliva and Magrini (3) reported that in normal fasting human beings the distribution in the blood was 150 mcg. per 100 ml. of plasma and 134 mcg. per cent in the corpuscles. However there is general agreement that blood values are o little value in determining the adequacy of a dietery supply either by basic levels or by response to test doses. Tissue content of coenzymes was found by Elvehjem (4) to be significant in contrasting normal and black tongue dogs. Seventy per cent lower liver content and 35 per cent lower striated muscle content appeared in the organs of dogs with black tongue. Such tests are naturally impossible on human beings.

TABLE 39

Suggested daily allowances protein and niacin

(Food and Nutrition Board, National Research Council, 1948)

Subjects	Calories	Protein	Niacin
		grams	*mg.*
Man			
Sedentary	2400	70	12
Moderately active	3000	70	15
Very active	4500	70	18
Woman			
Sedentary	2000	60	10
Moderately active	2400	60	12
Very active	3000	60	15
Last half pregnancy	2400	85	15
During lactation	3000	100	15
Children under 12 yrs.			
Under 1 yr	110/2.2 lbs.	3.5/2.2 lbs.	4
1–3 yrs	1200	40	6
4–6 yrs	1600	50	8
7–9 yrs	2000	60	10
10–12 yrs	2500	70	12
Boys and girls over 12 yrs.			
Boys			
13–15 yrs	3200	85	15
16–20 yrs	3800	100	17
Girls			
13–15 yrs	2600	80	13
16–20 yrs	2400	75	12

Pellagra Therapy

Pellagra treatment today is usually oral administration of 500 mg. of niacin or niacinamide daily, given in 10-mg. doses at hourly intervals. When administered parenterally the total daily dose may vary from 40–80 mg. dissolved in sterile physiologic saline solution and injected intravenously in divided doses. It is important, however, that pellagra patients get with their medication a well balanced diet. Even before the discovery of the specificity of niacin, diets had been worked out (5) to prevent the development of the disease. Today the niacin content of common foods has been determined and is available (6).

However, while dry niacin and niacinamide are quite stable to heat, there is a certain loss in food cookery: one third to one half is lost in steaming, frying and boiling, and 60 per cent in baking (7). This should be borne in mind when calculating the niacin content of foods as they reach the table.

The enrichment of white flour and bread with niacin and the proposed enrichment of corn products should go far toward reducing the incidence of pellagra.

Human Tryptophan Requirement

The lack of tryptophan in corn protein and its effect in increasing niacin requirement has already been explained. Holt et al (8) have reported this and suggest that 6–9 mg. of tryptophan per day is desirable, and at least 3–6 mg. on the basis of urinary excretion data. Infants appear to need nearly five times the adult requirement; 23–40 mg. per kilo of body weight.

In selecting pellagra preventive diets it is therefore important not only to meet the protein allowance but also to attend to quality (amino acid content) and to tryptophan content of the proteins in particular.

Intestinal Synthesis of Niacin

To what extent the body can meet its niacin need by intestinal synthesis and absorption of the synthesized niacin is a problem. Ellinger and Coulson (9) have shown that the urinary excretion of F_2 could be diminished by feeding sulfasuzadine or sulfaguanidine; that the diminution was not due to interference with niacinamide metabolism, and that this would indicate intestinal synthesis and absorption of the product. Najjar and Holt (10) have also given evidence of intestinal synthesis of the vitamin. The significance of such synthesis in estimating niacin daily needs is still uncertain. It may well be disregarded in building human diets.

Niacin and Domestic Animals

Cattle: As in the case of thiamine and riboflavin, rumen synthesis makes these animals practically independent of dietary supply of niacin after they are two months old. It has been reported (11) that calves need a niacin supply in the diet. Deficiency in calves is manifested by scours (diarrhea), occasionally bloody stools, dehydration. Acute deficiency is followed by death. In the advanced cases there are extensive petechial and ecchymotic hemorrhages in the mucosa of the stomach and duodenal portion of the small intestine. Clots of blood in the stomach and the remainder of the intestinal tract give the feces a red or reddish black color.

Sheep: Rumen synthesis appears adequate for sheep and even lambs on a low niacin intake for 8 months developed normally (12).

Swine: Niacin in the diet is a specific requirement for swine, at least during the growing stages. Lack results in growth retardation, occasional vomiting and foul smelling feces. For growing pigs the following are recommended daily allowances:

Live wt.	*Mg. Niacin Daily*
50 lbs.	7.0
100 lbs.	12.5
150 lbs.	16.5
200 lbs.	19.0
250 lbs.	21.0

TABLE 40

Microörganisms studied for use in microbiological assay of niacin

(After Snell (14) in Biological Symposia, **12:** 185, 1947)

Organisms	Introduced by	References
Proteus	Lwoff and Querido (1938)	(15)
Shigella paradysenteriae	Dorfman, et al. (1939)	(16)
Lacto-bacillus arabinosus	Snell and Wright (1941)	(17)
Lacto-bacillus casei	Landy and Dicken (1942)	(18)
Leuconostoc mesenteroides	Gaines and Stahly (1943)	(19)

Poultry: Chicks on a niacin deficient diet develop an inflammation of the tongue and mouth cavity, a sort of black tongue disease. Growth is retarded, feed consumption is reduced, and there is often poor feather development and a scaly dermatitis of feet and skin. Ag least 0.5 mg. per 100 grams of ration is necessary to prevent these symptoms (13).

Niacin has not been shown essential for mature fowls, but for starting chicks the recommended allowance is 8 mg. per pound of feed; of tryptophan, 1.8–2.3 grams per pound of feed.

Bacterial Requirement for Niacin

The specific requirement of coenzymes by the influenzae bacilli made possible the assay of these "V" factors. There are also several bacteria which because of their requirement for niacin or niacinamide for growth are being used in the microbiological assay for these vitamins (See Table 40).

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CHAPTER X. PYRIDOXINE OR VITAMIN B_6

SECTION I. FUNCTIONS OF PYRIDOXINE

In 1930 Chick and Copping (1) described a B-Complex factor to which they gave the provisional name of Vitamin "Y". This factor was subsequently rediscovered in 1934 by P. Gyorgy (2) who at the time thought it might be identical with Reader's (3) postulated B4. The same factor was called filtrate factor I by Lepkowsky et al (4) and vitamin H by Hogan and Richardson (5) and Booher (6). However, in 1935 Birch, Gyorgy and Harris (7) reported that a particular type of dermatitis which they called acrodynia or florid dermatitis was due to a specific vitamin. In 1936 Birch and Gyorgy (8) gave the details of its chemical nature and methods of concentrating the vitamin to which they gave the name of B_6.

The vitamin was isolated in 1938 and chemically identified in five different laboratories: by Gyorgy (9), Lepkowsky (10), Kuhn (11), Ichiba (12) and Keresztesy (13).

Kuhn and Wendt (11) suggested calling the vitamin "adermin" but in 1939 Gyorgy and Eckhardt (14) criticised this name and suggested the name pyridoxine for the following reasons:

> "Rats in which acrodynia has been produced by a B-free diet supplemented by vitamin B_1 and B_2 improved or recovered from a specific dermatitis when 10 micrograms of vitamin B_6, natural or synthetic, were added to the diet. But, in the further course of the experiments, many of the animals died from other internal deficiency diseases and later a large percentage of the remaining rats developed severe skin lesions of three more or less differing types, differing from those seen in acrodynia. Therefore the part played by vitamin B_2-Complex in dermatological conditions has to be extended beyond B_6 and the term "adermin" because it is misleading, should be abandoned. In accordance with the chemical nature of B_6, which is a pyridine derivative containing several oxy- or methoxy-groups the term "pyridoxine" appears to be appropriate."

Chemical identification was established when Harris and Folkers (15) synthesised the vitamin and confirmed the formula of Kuhn (16) and Keresztesy and Stevens (13).

Today, the term vitamin B_6 actually means a group of compounds. In assaying sources of vitamin B_6, Snell, Guirard and Williams (17) obtained widely different results from measurements by the yeast method and with the organism S. Lactis R. (S. fecalis R.). Assays with the latter organism led them to postulate a form of pyridoxine more active than pyridoxine itself which they tentatively called "pseudopyridoxine". Later Snell (18) showed that the pseudopyridoxine comprised two derivatives of pyridoxine, one an aldehyde or pyridoxal, and the other an amine or pyridoxamine.

Harris, Heyl and Folkers (19) determined their structure and synthesis, and subsequently showed transformation of pyridoxine into these forms gave them functions other than that possessed by pyridoxine itself.

Acrodynia or Florid Dermatitis

The dermatitis called acrodynia is characterized by symmetrical changes in the peripheral parts of the rat body, the paws, nose, mouth, and ears. Antopol and Unna (20), in a special study of the skin lesions associated with B_6 deficiency, state that the hyperkeratosis and acanthosis of the ears, paws and snout of the rat and the edema of the corium may be considered specific of B_6 deficiency.

However, it was early noted that acrodynia does not always develop in vitamin B_6 deficiency (21, 22), nor is it always cured by B_6 when it does occur (23). Further investigation revealed that at least two and perhaps more factors complementary to B_6 were involved in the prevention and cure of acrodynia. Numerous investigators have contributed to the evidence showing the involvement of essential fatty acids and pantothenic acid. Of these the report of Quackenbush et al (24) may be selected as illustration.

These investigators produced acrodynia in rats on a diet low in unsaturated fatty acids (.003 per cent). The value of pyridoxine, pantothenic acid, and ethyl linolate, alone and in various combinations, was studied with the following results:

1. Pantothenic acid alone did not cure the acrodynia; pyridoxine alone produced temporary alleviation but did not effect cure; ethyl linolate alone cured the acrodynia and in subcurative amounts became curative when supplemented with pyridoxine.

This confirmed the opinion of Birch, Gyorgy, and Harris (7) that essential fatty acids are necessary for the utilization of B_6 and vice versa. An acrodynia that results from a diet containing inadequate amounts of essential fatty acids and B_6 could be cured by either B_6 or essential fatty acid.

2. Pantothenic acid combined with B_6 improved the dermal conditions more than pyridoxine alone. Subsequent addition of ethyl linolate produced still further improvement. The three factors together cured the acrodynia but did not completely cure the scaly condition of the tail and hind paws. This suggested a need for still another dietary factor not identified at the time.

3. In prophylactic tests, neither pyridoxine not pantothenic acid prevented the acrodynia, but pyridoxine did retard the development of the dermal lesions. A deficiency of pyridoxine did not result in acrodynia when the animals were fed both linoleic acid and pantothenic acid: only a slight scaliness resulted.

4. Sustained growth resulted only when all the supplements (linoleic acid, pantothenic acid, and pyridoxine) were fed.

In the light of these and confirmatory findings acrodynia cannot be considered a specific pyridoxine deficiency.

Convulsions and Pyridoxine Deficiency

In 1940 Chick et al (25) reported convulsions in pyridoxine-deficient rats and pigs. These fits, which resembled epileptic seizures were prevented and cured by a dosage of 10–15 micrograms of B_6 daily. Fouts et al (26) reported similar effects in dogs and Wintrobe et al (27) and Hughes and Squibb (28) in pigs.

Daniel et al (29) have also described a convulsive syndrome in rats which they claimed was a specific result of pyridoxine deficiency. Also Lepkowsky et al (30), suggested that the epileptic-like fits might be related to a disturbance in water metabolism.

More recently Davenport and Davenport (31) have studied the problem by means of electro-shock threshold measurement. (Electro-shock threshold is the smallest amount of current required to produce a detectable clonic seizure). They found that a diet deficient in pyridoxine produces in growing rats a progressive diminution in weight gain and a tendency for the electroshock threshold to fall. Injection of pyridoxine caused a rapid and highly significant rise of electro-shock threshold in mildy deficient rats. This phenomenon suggested to them that even a mild degree of pyridoxine deficiency causes an increase in brain excitability.

The injection of the vitamin raised the threshold more slowly in rats on a diet severely deficient in pyridoxine. Their most interesting finding is that interference with transamination may be responsible for the effect of pyridoxine deficiency on brain excitability. By feeding glutamic acid to B_6-deficient animals they raised the electro-shock threshold and state:

> "A simple explanation of our findings is that extra glutamic acid promotes a more efficient utilization of pyridoxine in the transaminase system and that maintenance of normal transamination is essential for the tests of normal brain function employed in this study."

Blood Dyscrasias and Pyridoxine Deficiency

Pyridoxine deficiency in the dog (26) and in the pig (27, 31), results in the development of a microcytic hypochromic anemia. The hemoglobin and the red cells decrease progressively, the hemoglobin relatively faster than the red cells. There is an elevated plasma iron level, hemosiderosis in the liver, spleen, and bone marrow, and hyperplasia of the bone marrow. Pyridoxine deficiency anemia and pernicious anemia are similar in several respects; in both there is an increase in serum iron, hemosiderosis of the tissues, hyperplastic bone marrow, and neurologic lesions. They differ in that microcytosis characterizes the pyridoxine anemia and macrocytosis the pernicious type.

The condition has been studied in detail by Cartwright et al (33), who state that the ferremia and hemosiderosis are caused by the continued absorption or decreased excretion of iron at a time when its utilization for hemoglobin formation is at a minimum and when the tissues are abundant in iron. *There is a faulty synthesis of hemoglobin.* Cartwright and Wintrobe (59) stated later that the fundamental disturbance in B_6 deficiency anemia is failure to synthesize photoporphyrin. Smith et al (34) report that a similar anemia occurs in dogs. It responded specifically to pyridoxine but pyridoxine alone or in combination with other factors of the B-Complex was not sufficient to maintain the hemoglobin at normal levels. Brewer's yeast at a 10 per cent level maintained a high hemoglobin level therefore there appeared present in the yeast a factor in addition to B_6, which stimulates hemoglobin production in the dog. This factor might be folic acid.

A high incidence of another blood dyscrasia apparently caused by pyridoxine deficiency, abnormally shaped erythrocytes (poikilocytosis) was reported to occur in dairy cattle. This condition was accompanied by anorexia, thinness, unthriftiness, a dry and harsh condition of the hair coat, and a retarded growth rate in growing animals. Neither riboflavin nor niacin corrected this condition, but a daily dosage of 40 mg. of pyridoxine for three or four weeks brought improvement in health and an accelerated growth rate. Normally cattle synthesize enough B_6 in the rumen to meet their needs, but when this is inadequate, the poikilocytosis may result. It is unknown at present whether pyridoxine deficiency is responsible for what is known as "sickle cell" disease in man.

Muscular Dystrophy and Pyridoxine Deficiency

In 1939 Spies, Bean and Ashe (36) observed in four of their pellagra patients a syndrome characterized by extreme nervousness, insomnia, irritability, abdominal pain, weakness and *difficulty in walking*. These symptoms dramatically disappeared following intravenous injections of 50 mg. of synthetic pyridoxine. Later (37) they treated with equally satisfactory results some twenty similar cases and in these cases pyridoxine produced results not accomplished by niacin, riboflavin or thiamine. Relief of difficulty in walking suggested an effect of pyridoxine on muscular dystrophy.

Since Parkinson's disease (paralysis agitans) is a condition of muscular weakness and rigidity, pyridoxine has been tried for effect on this disease (38, 39, 40, 41). Both positive and negative results have been reported and controversy exists concerning its value in the treatment of this disease (42, 43).

Pyridoxine and the Nervous System

The convulsive seizures resulting from pyridoxine deficiency in rats, dogs and swine have already been described. (See p. 176.) In 1938 Wintrobe et al (44) described changes in the nervous system of swine characterized by degenerative changes in the peripheral nerves, in the dorsal roots, in the ganglion cells, and in the posterior column of the spinal cord. This they described as a sensory neurondegeneration involving axis cylinders as well as myelin sheaths. Both pyridoxine and pantothenic acid deficiencies appeared to be involved. These effects were further studied by Follis and Wintrobe (45). In this study they attempted the differentiation of the effect of pyridoxine and of pantothenic acid. Apparently the histologic picture produced by a prolonged deficiency of either vitamin is the same but the changes begin in different locations. In pyridoxine deficiency myelin degeneration of the peripheral portions of the sensory nerve and axis cylinder degeneration occur first. In pantothenic acid deficiency pronounced chromatolysis of the dorsal root ganglion cells was the first change and only subsequently did changes in the peripheral nerves take place. In both groups changes in the posterior column of the spinal cord was a late effect. The possible relation of these changes to myelinoclastic and polioclastic nervous disease effects in man was suggested.

Forms of Pyridoxine and Their Relation to Function

The discovery of pyridoxal and pyridoxamine has already been described. As was stated (18) the vitamin B_6 content of natural materials showed widely different results when measured by the yeast method in comparison with assay by *S. fecalis* R. The existence in natural products of at least three forms of pyridoxine; pyridoxine per se, pyridoxine aldehyde, and pyridoxine amine explained these results. Their structure (See Section II) was determined by Harris et al (19).

Pyridoxine itself and the two derivatives show by the yeast assay essentially the same activity as grouth stimulants, but pyridoxal and pyridoxamine showed 5,000 to 9,000 times the activity of pyridoxine for the growth of *S. fecalis R.* It is suggested that differences in the activity of these compounds for different organisms may be dependent on the relative ability of the organisms to change pyridoxine to the more active forms. It should also be noted that these three may not be the only forms of pyridoxine present in natural materials possessing B_6 activity. According to Hochberg et al (46) there is evidence that B_6 activity in natural materials is not limited to these three compounds, but that yeast may contain a labile B_6 form which behaves differently from these three forms.

Rapid progress has been made in the elucidation of the metabolic functions of vitamin B_6 forms. It has been shown that phosphorylated pyridoxal (47) serves as the essential prosthetic group of enzymes which decarboxylate tyrosine, arginine, glutamic acid, dopa, and other amino acids (48, 49). Apparently pyridoxine is first oxidized to pyridoxal which is then in turn phosphorylated by adenosine triphosphate (A.T.P.).

There is also evidence that phosphorylated pyridoxamine can function with enzymes that carry on transamination reactions. In 1945 Schlenk (50) showed that the tissues of rats deficient in B_6 were low in transaminase. Addition of pyridoxal and pyridoxamine plus ATP reactivated the deficient systems partially in the majority of trials. This was confirmead by Ames et al (51) and that phosphorylated pyridoxamine can function with transaminase enzymes was reported by Umbreit et al (52) and by Schlenk and Fisher (53). In 1945 Snell (60) demonstrated the interconversion of pyridoxal and pyridoxamine by heating with the appropriate amino or keto acid. He therefore suggested that the transfer of amino groups which occurs in transamination involves some change within the coenzyme molecule, viz.:

pyridoxal phosphate   pyridoxamine phosphate

This viewpoint was supported by the work of Umbreit et al (52) by whom pyridoxamine phosphate was found to be active as the coenzyme of the transaminase and by Ames (51) et al who found pyridoxamine phosphate stimulated the depressed transaminase activity of liver homogenates prepared from pyridoxine deficient animals.

According to Snell's hypothesis pyridoxal and pyridoxamine phosphates should be equally active in transaminase systems since they are interconvertible during coenzyme function.

This viewpoint has been questioned by later work of Umbreit et al (61). They noted that one of their transaminase preparations from S. fecalis R. and one from pig heart could not be activated by pyridoxamine phosphate while pyridoxal phosphate was active. They also put the pyridoxamine phosphate to a more critical test by using a more purified transaminase preparation; one freed of coenzyme and other enzymes which might effect a conversion of pyridoxamine to pyridoxal. The glutamic-aspartic transaminase of pig heart was purified and the formation of oxalacetate from aspartic acid and alpha-keto glutarate used as a test of its activity. The pyridoxamine phosphate was prepared by heating pyridoxal phosphate with glutamic acid. In this test pyridoxal phosphate acted as a coenzyme but pyridoxamine phosphate failed to do so; that in spite of the fact that

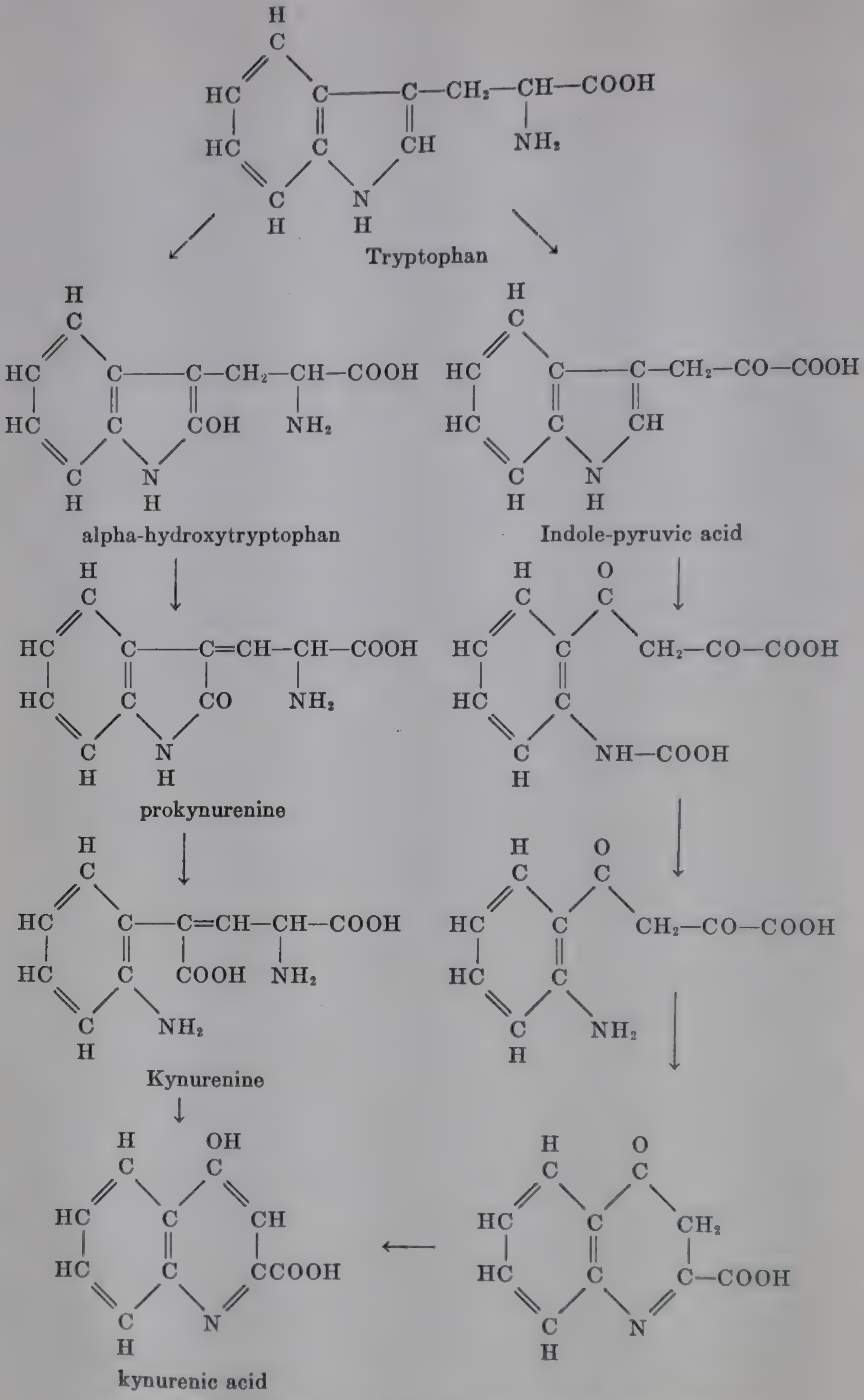

Fig. 19. Possible Steps in the Metabolism of Tryptophan

its growth effect was like that of pyridoxamine phosphate prepared by direct phosphorylation of pyridoxamine.

Pyridoxine and Tryptophan Metabolism

In 1946 Umbreit et al (54) reported that pyridoxal phosphate acts in the formation of tryptophan from indole and serine. They obtained from the frozen mycelium of a strain of neurospora a cell-free extract which catalyzed the reaction:

indole plus serine → tryptophan plus water

According to Tatum and Bonner (55) the well known formation of indole from tryptophan in *E. coli* is the reversal of the above reaction.

The metabolism of tryptophan in animal tissues is known to be profoundly altered by pyridoxine deficiency. In 1942 Lepkowsky and Nielsen (56) noted that the urine of pyridoxine deficient rats, when treated with iron sulfate, yielded a green pigment. The precursor of this green pigment was later identified (57) as xanthurenic acid, a product of tryptophan metabolism. Kynurenine has also been recognized as a product of tryptophan metabolism, probably an intermediate in the formation of xanthurenic acid (58). Cartwright et al (27) measured the xanthurenic and kynurenic acid excretion in swine and found that these products varied with tryptophan and pyridoxine intake. Reid et al (58) studied the excretion of xanthurenic acid in pyridoxine deficient rats with the purpose of determining the pathway of tryptophan to xanthurenic acid metabolism. They tried d- and l-tryptophan; pyruvic, lactic, acetic, and propionic derivatives of indole; kynurenine, kynurenic acid, and indole and serine. Of these only l-tryptophan and kynurenine appeared to yield xanthurenic acid and the excretion of the latter was diminished by administration of pyridoxine. The pathway apparently:

tryptophan - kynurenine - xanthurenic acid

Just how B_6 functions in this tryptophan metabolism is still uncertain, but it appears safe to claim that pyridoxal phosphate plays an essential role in the normal animal metabolism of tryptophan, possibly by hydrolytic removal of the side chain of tryptophan. (See figure 19.)

Summary

The functions of pyridoxine described have been those demonstrated in certain animals and micro-organisms. Its relation to human nutrition is discussed in Section III.

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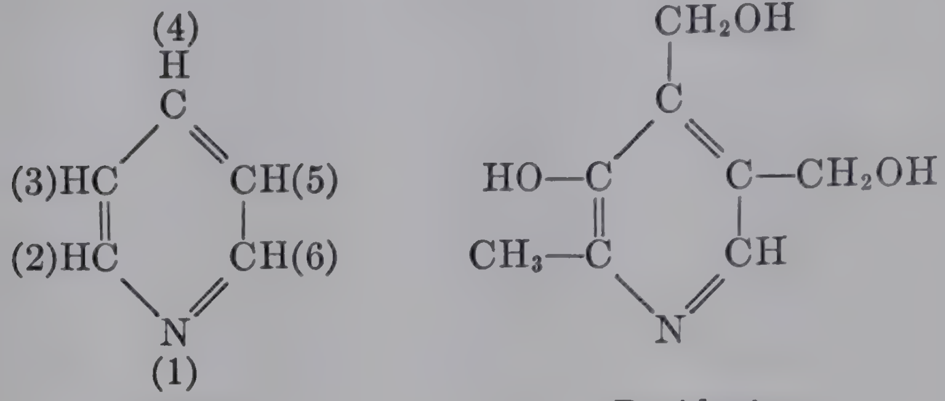
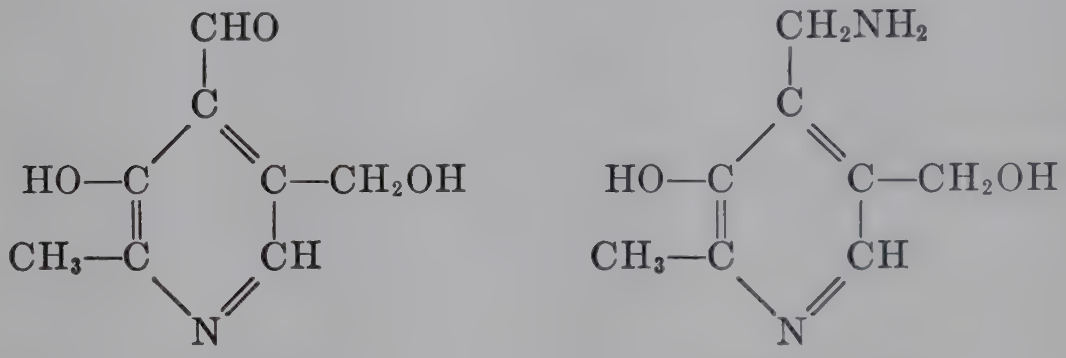
SECTION II. FORMS AND CHEMISTRY OF VITAMIN B_6

The structure of vitamin B_6 and its properties was established by several laboratories in 1938 and 1939 (1, 2). At the time only one form was known but the discovery of "pseudopyridoxine" by Snell, Guirard and Williams (3) in 1942 inaugurated a search for other forms of the vitamin. These investigators reported that the high activity of tissue extracts in promoting growth of *S. fecalis R* on a pyridoxine-free medium was due to a naturally occurring, physiologically active metabolite that could be formed by the organism from pyridoxine. This metabolite, tentatively named "pseudopyridoxine," had for that organism many times the activity of pyridoxine itself.

But pseudopyridoxine proved to be two compounds, one a pyridoxine aldehyde now called pyridoxal, and a pyridoxine amine now known as pyridoxamine. These compounds were successfully synthesized by Harris, Heyl and Folkers (4) in 1944. The structure of these three forms is shown in Figure 20.



Pyridine Pyridoxine



Pyridoxal Pyridoxamine

FIG. 20. FORMS OF VITAMIN B6

Pyridoxine

In 1938 Keresztesy and Stevens (5) reported the isolation of vitamin B_6 as the hydrochloride and also certain of its properties. In 1939 Stiller, Keresztesy, and Stevens (6) gave the data on which they based determination of its chemical structure, and Harris, Stiller and Folkers (7, 8) gave details of the synthesis of the vitamin. Other workers (9, 10, 11, 12, 13, 14, 15) confirmed their findings. The vitamin was proven to be a derivative of pyridine [2-methyl-3-hydroxy-4.5 di(hydroxy-methy;) pyridine].

Pyridoxine is sold in the form of white platelets melting with decomposition at 206-208° C. One gram dissolves in 4.5 cc. of water and in 90 cc. of alcohol. Aqueous solutions have a pH of 3.2.

The compound is stable in concentrated HCl at high temperatures but is destroyed by light and by ultraviolet irradiation. It is not affected by heating with alkalies, nitrous acid, ethyl nitrate, or Fehling's solution.

Absorption spectrum studies (6) indicated that the compound had tautomeric properties, the absorption maxima varying with the hydrogen ion concentration, e.g.

> In acid solution at pH 2.1 a single band at 2920 Angstrom. Between pH 4 and 5.1 two new bands appear at 2550 and 3260. At pH 6.75 the acid band (2920) disappears and the 3260 band has fully developed. Between pH 6.75 and 10.2 the 2550 band has fully developed but both alkaline bands exhibit a shift toward 2460 and 3110 respectively.

Pyridoxal and Pyridoxamine

In 1944 Snell (16) gave details of the treatments of pyridoxine which produced pyridoxal and pyridoxamine and accounted for the variations in activity for microorganisms. Some of the data on which he based the following conclusions are shown in Table 41.

1. For the production of pyridoxal he found that only permanganates, chromates, dichromates, and MNO_2 effective; $KMnO_4$ effective in alkaline, neutral or weakly acid solution, MnO_2 only in acid solutions.

2. Ammonia proved the most effective for production of pyridoxamine and pyridoxine esters were more readily activated than the non-acetylated form.

3. Pyridoxal and pyridoxamine are reversibly convertible one to the other according to the following types of reactions:

a) $RCH_2OH \rightleftharpoons RCHO \rightleftharpoons RCH{=}NH \rightleftharpoons RCH_2NH_2$

b) Pyridoxal + glutamic acid $\rightleftharpoons$ pyridoxamine + alpha-keto glutaric acid.

4. Of the action of the individual amino acids:

a) Glutamic acid and lysine effect complete conversion of pyridoxal to pyridoxamine.

b) Methionine, l-tyrosine, dl-phenyl alanine, l-aspartic, l(+)arginine, l-leucine, dl-isoleucine, dl-alanine, dl-valine, dl-threonine, l-cystine, glycine effect partial conversion.

c) l-hydroxyproline, l-proline, dl-serine, beta alanine produce no reaction.

d) l-tryptophane, l-histidine destroy activity.

In differentiating activity products, use is made of 3 micro-organisms; *S. fecalis R.*, *L. casei*, and yeasts. In general pyridoxine, pyridoxal and pyridoxamine show the same activity for most yeasts; pyridoxal activity for both *S. fecalis R.* and *L. casei*; pyridoxamine great activity for *S. fecalis R.*, but less for *L. casei*.

TABLE 41

Properties of pyridoxal and pyridoxamine

After Snell (16) (17) 1944–45)

I. Treatments that affected Pyridoxine Activity

Treatment	Activity compared with pyridoxine as 1.0
Heated with ammonia	22.0
Heated with cystine and NaAc	10.6
Acetylated with acetic anhydride in pyridine	20.0
Oxidized with $KMnO_4$	160.0

II. Relative potency of pyridoxine and pyridoxine ester

Compound	Activity for:		
	S. fecalis R.	L. casei	S. cerevisiae
Pyridoxine	1.0	1.0	1.0
Acetylated pyridoxine	7.0	4.7	1.1

III. Conversion to pyridoxamine by treatment of pyridoxine ester

Treatment by	Activity compared with pyridoxine as 1.0
Ammonia	80–400
Ammonium carbonate	200
Methyl amine	1.0
Trimethyl amine	1.0
Sodium carbonate	1.0

IV. Relative effect of oxidants

Oxidant	Amount B_6	Amount oxidant	Temperature	Time	
	mg.	*mg.*	°C.	*minutes*	
$KMnO_4$	1.0	0.3	25	30	
$KMnO_4$	1.0	1.0	25	30	
$KMnO_4$	1.0	10.0	25	30	
$K_2Cr_2O_7$	1.0	1.0	100	15	
MnO_2	1.0	5.0	100	15	

Snell and Rannefeld (18) made a special study of the effect of the three forms on some 17 different organisms with the following results:

1. Equal activity on a molar basis for all three on yeasts, molds, and rats, but certain yeasts got slightly less growth effect from pyridoxamine.

2. A large group of lactic acid bacteria increased growth on pyridoxamine and pyridoxal but apparently did not utilize pyridoxine at all.
3. For *S. fecalis R.* pyridoxamine is highly active, pyridoxal less active pyridoxine almost inactive.
4. For *L. casei* pyridoxal is highly active, pyridoxamine less active.
5. For *S. carlsbergensis* all three were equally active.

Pyridoxal and pyridoxamine were sucessfully synthesised by Harris, Heyl, and Folkers (19) and the following properties have been reported (18):

Both undergo variable changes when autoclaved with the nutrient medium, when heated in water containing dissolved air, when exposed to light.

Pyridoxamine is destroyed by nitric acid; pyridoxal and pyridoxine are stable in its presence.

Pyridoxal is inactivated by NaCN and NH_4Cl and by standing with acetone in alkaline solution.

Heating with casein hydrolysate destroys activity of pyridoxal for *L. casei* and increases activity for *S. fecalis R.*, presumably by conversion into pyridoxamine.

Pyridoxamine is converted to pyridoxal by heating with alpha-keto glutaric acid. (See Table 41.)

The B_6 activity of liver and yeast extracts appear due mainly to their pyridoxamine and pyridoxal content; of rice bran to pyridoxine itself.

Cunningham and Snell (20) also report the following additional properties:

That all three forms are destroyed by exposure to light; destruction more rapid above pH 7 and all more stable in 0.1 N acid.

That none of them are destroyed by heating with 5 N sulfuric or hydrochloric acids, but that pyridoxamine shows a slight breakdown in the presence of alkali; not so pyridoxine or pyridoxal.

That all three are rapidly destroyed by oxidizing agents.

Replacement of B_6 by an Amino Acid

Snell (21) has reported that enzymatic digested vitamin-free casein contains a factor which together with dl-alanine permits the growth of *L. casei* in the absence of any form of B_6. In the presence of this unidentified casein factor d(−) alanine promotes growth of *L. casei* but l(+) alanine is inactive; and both d(−) alanine and l(+) alanine can replace B_6 for *S. fecalis R.* Snell and Gurrard (21) have suggested that alanine may be a precursor of pyridoxine.

Antipyridoxine

As in the case of other vitamins there exists an analog of pyridoxine that is antagonistic to its action. This compound is (2, 4 di-methyl-3-hydroxy-

5-methylol pyridine) or desoxypyridoxine. Ott (22) has also reported that for chicks 2-methyl-3 hydroxy-4 methoxy methyl-5 hydroxy methyl pyridine has the same order of anti-pyridoxine activity as desoxypyridine.

Ott (22) demonstrated that when this compound was fed to chicks it produced a disease that could be prevented by increasing the pyridoxine content of the ration. In rats it caused atrophy of the thymus and attendant reduction in antibodies.

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Section III. Evaluation for Human and Animal Nutrition

Vitamin B_6 is required in the diet of all animals so far investigated. It would be reasonable to expect that it would also be an essential of human diets and that probability is not yet eliminated. However, the Council on Pharmacy and Chemistry of the American Medical Association has stated (1):

> "Pyridoxine has been available for too brief a period and in insufficient quantities to permit its clinical evaluation. Further study of the clinical value of this compound is necessary before definite claims will be permitted."

Nutrition Reviews in April 1947 (2) summarized the situation as follows:

> "It is natural that pyrodoxine should be tried clinically in conditions showing points of similarity to symptoms of deficiency in animals. Therapeutic trials on patients with muscular weakness, epilepsy, certain types of dermatitis, and with nausea and vomiting of pregnancy have been made. In only a few cases have positive results been reported. No human counterpart of the animal anemias produced by vitamin B_6 deficiency has been recognized. Much more work, of a carefully controlled nature, is required to evaluate the usefulness of this vitamin in medical practice."

An Experiment on Human B_6 Deprivation

Hawkins and Barsky (3) have reported a study aimed to evaluate the significance of B_6 in human nutrition. This experiment is of particular interest since it was not treatment of a disease symptom, but rather a measure of what may happen when vitamin B_6 is eliminated from the diet of a normal human being. Hawkins subsisted for a period of two months on a purified diet containing all the known necessary vitamins except vitamin B_6. The basal diet was a mixture of sucrose, corn oil, vitamin-free casein, mineral oil and a cod liver oil concentrate. Throughout most of the experimental period the diet supplied 2178 calories daily; derived 58.5 per cent from carbohydrate, 26.3 per cent from fat, and 15.2 per cent from protein. The average protein intake was 85.2 grams daily and 50 mg. of iron per day. In addition definite amounts of B_1, calcium pantothenate, niacin, riboflavin, ascorbic acid, choline chloride, and inositol were fed daily, and an extract of B_6-free rice polish extract was included in the diet.

According to these authors, throughout the experiment there was no evidence of any marked disturbance in nitrogen balance; there was no significant change in the total and non-protein or amino nitrogen content of the blood. If B_6 is essential to protein metabolism in man it did not manifest any relation in this test.

Particular interest attaches to the effect on the blood picture and to the fact that near the end of the period there was an unusual degree of depression and mental confusion, the latter disappearing with B_6 supplementation.

The alteration in the blood picture is summarized in Table 42. There was definite alteration in the white blood cell picture involving the lymphocytes and neutrophils, but no changes in other blood cells. A similar change in these white blood cells has been reported to occur in monkeys on a B_6-free regimen (4). There have also been reported in some cases of pernicious anemia (5) and agranulocytic angina (6) rather rapid increases in neutrophils after intravenous administration of pyridoxine.

The authors of this paper state their conclusions as follows:

> Our experiment revealed that on a purified diet, over a period of two months, without vitamin B_6, no changes occurred which could unequivocally be considered as re-

sulting from a lack of these compounds. There is, however the possibility of albuminuria, of mental symptoms, and of white blood cell changes.

A longer experimental period would have been desirable. In the case of monkeys on essentially the same regimen (4) months were required to produce B_6 deficiency symptoms."

This paper commands special attention, as the claims for B_6 requirement in humans has been based largely on clinical evidence. A more recent study of the effect of B_6 deficiency on the monkey has been reported by Greenberg and Rinehart (28). The basal diet used was a modification of the M3 diet used by Waisman and McCall (29) and especial attention was given to blood changes.

TABLE 42

Effect of B_6 deficiency on the human blood picture

(After Hawkins and Barsky (3) 1948)

	Blood N.P.N.	Hb	Leucocytes, total per mm.	Lymphocytes	Neutrophils
A. While on B_6-free diet					
	mg. per cent	*gm. per cent*		*per cent*	*per cent*
At start of experiment.......	33.6	16.0			
5th day....................			7400	27	67
19th day....................	32.6	16.1			
38th day....................	32.5	15.4	6900	38	57
54th day....................	34.0	15.8	8800	53	40
B. After supplementing with 10 mg. B_6 daily					
5th day....................	32.6	14.2	8990	36	59
7th day....................		14.8	7600	31	65
C. After return to ordinary diet					
38th day....................		15.1	7720	35	60

They describe their results as follows: Within 2 to 3 weeks after withholding the vitamin from the diet the monkeys ate less food and began to lose weight but showed little change in appearance until 6–9 months after B_6 deprivation when they became sluggish in movements and hyperirritable. They also developed edema around the eyes; conditions also noted by the Wisconsin observers (4).

Most of the animals also showed hair changes consisting of thinning in some, bald patches in others and slight greying in still others. The majority developed fissuring and cracking of the epidermis of the hands after some 3–6 months on the dietary regime. A constant finding was the development of a slowly progressive anemia.

During the experiment the B_6 content of the blood was studied. When first brought to the laboratory the levels were usually below 5mcg/100ml but after 2–3 weeks on the complete diet levels increased to 5–25 mcg/100ml. Within a week or two after withdrawal of the vitamin from the diet the blood B_6 had dropped below 5mcg/100ml and in 2–3 weeks to 3–2 mcg/100ml. In long standing deficiency as low as 1mcg/100ml. Controls on a daily intake of 1 mg B_6 ranged from 5.0–20.8mcg/100ml, average 11.2.

The vitamin was present in both plasma and cells; on a good intake the plasma content is usually higher than the cell content but in the B_6 deficient monkeys there was nearly equal distribution between plasma and cells. Alterations in intake appeared to influence the plasma concentration more rapidly than cell concentration.

There was wide variation in the blood values of animals receiving B_6 which they could not explain. It may be that on a constant daily intake increase in size accounts for the progressive decreases in blood concentration. In Section I (p. 177) experiments on its value in certain muscular dystrophies and the conflicting results were reviewed. Some other clinical reports are reviewed in the following pages.

B_6 and the Nausea and Vomiting of Pregnancy

Several investigators (7, 8, 9, 10) have reported relief of the nausea of pregnancy by oral or intravenous dosage with pyridoxine in amounts from 2–200 mg. According to these reports, 50 mg. given three times daily by mouth or by vein frequently eliminated or greatly reduced the severity of the nausea and vomiting. Hesseltine (11), however, was unable to confirm these results. He treated 16 cases and to some gave placebos. Of the 11 treated cases given 100 mg. daily by mouth and 100 mg. twice weekly intramuscularly results were good with only 3, fair with 3 others, and poor with 5. 3 of the subjects on placebos also improved.

It is of interest however that six other groups of investigators (12, 13, 14, 15, 16, 17) have presented data which when combined indicate that the nausea and vomiting of radiation sickness was eliminated in 232 out of 313 cases by use of pyridoxine. Reeves (14) suggested that 50 mg. be given by mouth in the morning (about four hours before treatment), 25 mg. at noon, and 25 mg. after the evening meal.

In these cases the query arises as to whether the effects are pharmacological, a sedative effect of the drug, rather than correction of a dietary deficiency.

B_6 and Human Skin Lesions

No human counterpart of the acrodynia of the rat in man is known. However in acne there are seborrheic eruptions and the possible relation of pyridoxine to fat utilization stimulated tests of the use of the vitamin on acne. Here again, however, reports are conflicting. Jolliffe et al (18) in 1942 reported that 50–250 mg. of pyridoxine daily in divided doses produced good results in two series of cases, and in 7–10 days of treatment. Also certain cases showed a reduction of skin oiliness independently of the acne.

However, Stillians (19) was unable to duplicate these results. Only 3 out of 27 of his patients treated with 50 mg. or more of pyridoxine daily showed any striking improvement during a three months period of observation. Generally discouraging, or at least equivocal, results have been reported of the use of pyridoxine in the treatment of seborrhea (20, 21) vulvitis (22), and eczema (23).

The Requirements of Domestic Animals

All animals thus far investigated appear to require vitamin B_6. In the case of cattle and sheep, ordinarily, requirements are satisfactorily met by rumen synthesis. Cow's milk, according to assay by Schneider et al (24) contains 40 rat units per 100 grams (the rat unit being defined as the amount necessary to cure moderately severe acrodynia in three weeks). This should ordinarily be sufficient to meet the needs of the calf before weaning.

However, as already noted, Reid, Huffman and Duncan (25) reported a high incidence in dairy cattle of abnormal blood cells (poikilocytosis) accompanied by anorexia, thinness, unthriftiness, dry and harsh condition of the hair and a retarded growth rate in growing cattle. They attribute this to pyridoxine deficiency and inability to produce adequate pyridoxine by rumen synthesis.

Swine: Lack of pyridoxine in the diet of pigs can result in epileptic-like fits, microcytic anemia, low hemoglobin, and slowing of growth. The Committee on Animal Nutrition of the National Research Council (26) recommends a daily intake for growing and fattening pigs of 1.6 mg. for 50 lb. pigs; 3.0 mg. for 100 lb. pigs.

Poultry: Chicks on a pyridoxine-deficient diet show a small initial gain, then cease to grow or grow very slowly. They may show abnormal excitability and jerky convulsive movements; run about aimlessly, often flopping the wings and keeping the head down. Later, convulsions occur and during these the chick may rest on its breast, raise its feet off the floor and flop its wings, or it may fall on its side or roll over on its back and rapidly paddle its feet (bicycling). The head often jerks up and down or retracts much as in B_1 deficiency. Complete exhaustion follows one of these convulsions and is frequently fatal.

In mature birds pyridoxine deficiency is characterized by loss of appetite, followed by rapid loss of weight and death. Egg production and hatchability are also reduced.

The Committee on Animal Nutrition (27) recommends for starting chicks and for laying and breeding hens 1.6 mg. of pyridoxine per pound of feed.

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CHAPTER XI. PANTOTHENIC ACID

SECTION I. FUNCTIONS OF PANTOTHENIC ACID

Pantothenic acid was identified and named by R. J. Williams (1) in 1933. Of this discovery Williams (2) states:

> "Very briefly, the existence of pantothenic acid was first noted as a "yeast growth" substance; and its discovery had its basis in the work in Ide's laboratory in 1901, where a hypothetical substance, "bios", necessary for normal yeast multiplication was discovered. If the term "bios" were now to be used to designate any single chemical entity, it would most appropriately be applied to pantothenic acid, because, of all the substances known to be stimulatory to yeast growth, pantothenic acid is effective under conditions most nearly resembling those used in the Belgian laboratories."

Williams called it pantothenic acid because it was present in extracts of tissues representing many different biological groups. The name is derived from Greek words signifying "from everywhere."

In 1930–31 Ringrose et al (3) described a type of dermatitis in chicks, called temporarily "chick pellagra", curable by autoclaved yeast. The occurrence of a similar dermatitis was reported by Kline et al (4). The preventive factor was designated as filtrate factor II. by Lepkowsky and Jukes (5). Confirmation of the identity of pantothenic acid and filtrate factor came in 1939 (6, 7).

Pantothenic Acid as a Micro-organism Nutrilite

As already noted, the potent activity as a yeast growth stimulant led to its discovery by Williams and collaborators (1). Knight (8) has listed the organisms shown in Table 43 which are known to require pantothenic acid for growth. This fact has been used to devise a micro-organism assay method for the vitamin; Lactobacillus casei being most commonly employed (9).

Chick Pellagra

Pantothenic acid appears essential to maintain a normal skin, central nervous system, and a normal rate of growth in the chick. The manifestations of the so-called chick pellagra have been described as follows:

> "Margins of the eyelids become granular and a viscous exudate is produced that frequently sticks the eyelids together. Crusty scabs appear at the corners of the mouth which gradually enlarge to involve the margins of the skin around the nostrils and underneath the lower mandible. Skin at the bottoms of the feet and between the toes gradually thickens and cornifies and small cracks and fissures appear which enlarge and deepen and occasional hemorrhage may occur. Severely affected chicks become sensitive to walking."

Phillips and Engel (10) have described the effect on the nervous system of such chicks. There are spinal cord lesions consisting of myelin degeneration of the fibers distributed throughout the white matter of the cord, except in the posterior region, and there is chromatolysis of the Nissl material in the brachial segment nerve cells. In their experiments, if a pantothenic acid concentrate was given without riboflavin, the spinal changes did not occur, but a neuromalacia characterized by myelin swelling, degeneration, clubbing, and fragmentation of the axis cylinders invariably appeared in the sciatic nerves. If both pantothenic acid and riboflavin were administered no changes were evident in either spinal cord or sciatic nerve. Niacin and thiamine were ineffective in preventing cord changes.

TABLE 43

Bacteria that require pantothenic acid for growth

(After B. C. J. G. Knight (8) 1946)

Lactic acid bacteria
- Lactobacillus casei
- Bacillus lactic acidi
- Lactobacillus arabinosus
- Lactobacillus pentosus
- Lactobacillus delbruckii
- Bacillus brassicae
- Streptococcus lactis
- Leuconostoc mesenteroides
- Streptobacterium plantarum

Propionic bacteria
- Propioni bacterium pemtosaceum
- Propioni jensenii-1
- Propioni jensenii-29
- Propioni thionii-15
- Propioni petersonii-20
- Propioni technicum-22

Streptococci
- Streptococcus hemolyticus Richards (Lancefield A)
- Streptococcus hemolyticus Dochez N. Y. 5
- Streptococcus epidemicus Strains X40, X32, Cio8, W 116-7
- Streptococcus pyogenes
- Streptococcus zymogenes
- Streptococcus fecalis
- Streptococcus salivarius

Other microörganisms
- Pasturella hemorrhagica septicemia (13 strains)
- Proteus morganii
- Pneumococcus, strains of Types I, II, V, VIII
- Shigella paradysenteriae (Flexner) certain strains
- Clostridium tetani
- Clostridium welchii (perfringens) SR 12
- C. diphtheriae (certain gravis strains)

In a few cases the livers of the chick showed hydropic and fatty degeneration and in several cases the thymus was in an advanced state of involution if both pantothenic acid and riboflavin were inadequate.

The Committee on Animal Nutrition of the National Research Council (11) adds to the list of changes above growth retardation, ragged feather development, and in mature fowls a lowered hatchability of the eggs.

Achromatrichia

Human interest in pantothenic acid was stimulated by report of its ability to restore natural hair color and the premature advertising of calcium pantothenate for that purpose. Graying hair in black rats as a result of maintenance on a diet deficient in the B-Complex was first reported by Morgan Cook and Davison (12) and Lunde and Kringstad (13) in 1938. Also it was reported to be preventable and curable by use of filtrate factor. Later several other vitamins, viz. paba (14), biotin (15) and folic acid (16) were added to the list of those concerned with control of hair coloring and melanin deposition. The effect of its use on human hair restoration was studied by a number of investigators with conflicting results (17, 18, 19). In 1946 Nutrition Reviews summarized the situation to date as follows:

> "Pantothenic acid was at one time offered to the public as a specific against human achromatrichia (graying of hair). Carefully controlled observations, however, have shown that it has little or no value for that purpose."

Effect of Pantothenic Acid Deficiency in Animals

The effects of deficiency have been demonstrated in the following animals:

In the rat: omission from the diet results in a striking graying of hair, growth failure, and hemorrhage and necrosis of the adrenals; also anemia and leukopenia and porphyrin staining of the fur and whiskers.

In the dog: pantothenic deficiency manifests itself as a severe glycosemia, convulsions and coma, fatty livers, mottled thymus, and gastro-intestinal disorders including gastritis, enteritis, intussusception, loss of motility and decrease in the digestion and absorption of protein and carbohydrate; also a mild anemia.

In swine: there are nerve changes similar to those produced by pyridoxine deficiency but differing in certain respects. These differences were discussed in the chapter on pyridoxine (p. 178).

In poultry: the changes already described.

Hair changes similar to those in black rats have also been observed in pantothenic acid-deficient foxes.

Effect on the Adrenal Glands

In 1940 Daft et al (21) reported that adrenal hemorrhage and necrosis occurred in almost 100 per cent of the rats on a pantothenic acid-deficient diet; The condition was prevented and cured by synthetic pantothenic acid. In 1943 Ralli and Graef (22) reported that the removal of the adrenals (adrenalectomy) would restore the hair color lost through pantothenic acid deficiency. Following adrenalectomy there was transitory hyperplasia of the hair bulbs and increase in the deposition of melanin. This effect was confirmed by Butcher and Richards (23).

In 1945 Ralli and Graef (22) also reported on the effect of daily injections of desoxycorticosterone acetate or a natural extract of adrenal cortex hormone following an adrenalectomy. They found that it prevented the effect of the adrenalectomy on hair coloring; the desoxycorticosterone acetate being more effective than the natural extract.

Because it was known that the adrenal hormones are concerned in the excretion of salt (NaCl) and water, Spoor and Ralli (21) experimented and found that water deprivation or 2 per cent NaCl produced skin changes resembling those of pantothenic acid deficiency.

The explanation of the effect of pantothenic acid deficiency on hair color and on water metabolism therefore seemed to be related and correlated with its effect on the adrenal cortex. As a result a series of investigations were conducted to explain the method of action. Several investigators reported (24, 25, 26), that in pantothenic acid deficiency lipoid depletion of the adrenal cortex zona fasciculata was accompanied by great diminution in the weights of the thymus and lymph nodes, and that treatment with pantothenic acid produced a prompt and striking return of adrenal cortical lipids and a return to normal of thymus and lymph glands.

Deane and McKibbin (24), in attempting to correlate pantothenic acid deficiency with adrenal cortical function, suggest that the anatomic changes observed are explicable on the basis of increased functional activity of the cortex. They point out that cortical lipid depletion and involution of lymphoid tissues are non-specific alterations producible by "alarm stimuli" (27). They regard pantothenic acid as comprising a stimulus to increased production and release of cortical lipids with eventual exhaustion of the lipoid content of the gland.

Another explanation offered (28, 29) is that pantothenic acid deficiency produces insufficiency of adrenal cortical hormone. The two viewpoints are not necessarily irreconcilable. It may be that the "alarm" reaction is the initial response of the animal to pantothenic acid deficiency (hypercortical action), but when the response is sufficiently prolonged, adrenal exhaustion occurs followed by a terminal state of adrenal cortical hormone insufficiency (cortical hypofunction). The problem is complicated by a possible correlation of pantothenic acid deficiency and riboflavin deficiency.

Starting with the fact that hypofunction of the adrenal cortex in animals and man results in marked differences in water diuresis and resistance to water intoxication, Guant, Liling and Mushett (29) studied the effect of pantothenic acid deficiency and riboflavin deficiency on resistance to water intoxication. Riboflavin-deficient rats do not show adrenal lesions.

Their procedure with the pantothenic acid-deficient rats was as follows: Rats were placed on the deficient diet at the age of 3 weeks and after 4 to 6 weeks on the diet the animals were used for experiment. To test resistance to water intoxication either 5 or 13 doses of water (each dose 5 ml. water per 100 grams of body weight) were given by stomach tube at half hour intervals. (N. B., The lower dosage does not produce intoxication, the larger dosage does.) In every case the pantothenic acid-deficient animals excreted water less rapidly than the normal controls and succumbed in greater numbers to water intoxication. Furthermore normal diuresis and and efficient resistance to water intoxication were restored by administration of two doses of 1.0 mg. of calcium pantothenate by stomach tube eighteen hours and one half hour before the test. But two doses of 1.5 mg. each. desoxycorticosterone in 0.15 ml. of peanut oil at the same time periods (18 hours and $\frac{1}{2}$ hour before test) plus 0.5 ml. of adrenal cortical extract given with the third and sixth dose of water completely restored normal diuresis and resistance to water intoxication.

These observations appear to demonstrate that pantothenic acid deficiency impairs the mechanism by which the cortical hormone is formed, and creates a cortical hypofunction which is responsible for the change in water excretion and resistance to water intoxication and perhaps indirectly to the failure of melanin deposition. This conclusion, however, is not definite because similar deviations from the normal water metabolism occur in riboflavin deficiency, deviations preventable by both riboflavin and cortical hormones. Yet in riboflavin deficiency there are no adrenal lesions. Until the correlation of pantothenic action and riboflavin activity is explained the problem is not fully clarified. It is known, for example, that feeding pantothenic acid to deficient human beings restores not only the blood level of pantothenic acid but also the riboflavin level in those also deficient in that vitamin.

Pantothenic Acid as a Coenzyme

It was early shown that animal tissues contain very little free pantothenic acid. Incidentally the free acid is unstable, hence it is merchandised as the calcium salt or calcium pantothenate. It appears to exist in the tissues mainly bound to protein, requiring breakdown of this complex by enzymatic or other action to free it from the protein binding. This suggests that pantothenic acid might exist in the tissues as the prosthetic group of an enzyme system or coenzyme and that appears confirmed by present evidence; it exists in tissues as part of a coenzyme concerned in acetylation (coenzyme A).

In 1942 Wright (30) noted that production of hyperglycemia in rabbits also produced a 20–30 per cent decrease in the blood content of pantothenic acid, which suggests a possible participation of the vitamin in glucose metabolism. In the same year Berkman et al (32) reported that the microorganism *Proteus morganii* grown on a pantothenic acid deficient medium showed decreased ability to oxidize pyruvic acid or lactic acid; that addition to the medium of traces of pantothenic acid increased the ability of the organisms to oxidize both these acids.

Progress toward identification of the protein bound pantothenic acid as a coenzyme was accelerated in 1946 and 1947 by the studies of Lipman and coworkers. In 1945 Lipman (33) had shown the necessity for a coenzyme to produce the acetylation of sulfanilamide by a liver system. In 1946 two other groups of investigators (34), and (35), also reported the necessity of a coenzyme for the acetylation of choline.

The pantothenic acid coenzyme has actually been isolated and purified by Lipman and his coworkers (36). Their best preparation contained 11 per cent pantothenic acid, 9 per cent phosphorus, 18 per cent adenine, 22 per cent pentose, and some cystine, possibly in polynucleotide structure. The pantothenic acid content closely paralleled the coenzyme activity; a constant ratio of 0.7 mcg. of pantothenic acid per unit of coenzyme activity.

In breaking down the coenzyme, the combined action of a phosphodiesterase and an unidentified pigeon liver enzyme was used. Either, separately, destroyed the activity of the enzyme, an effect suggesting that two different linkages were broken to free the pantothenic acid. Incidentally, Nielands and Strong (37) have shown that this liver phosphatase combination when used in preparing material for pantothenic assays gives approximately three times as much liberated pantothenic acid as the digestion method usually used in assays reported in the literature. Hence, actually, the pantothenic acid content of products reported to date may be considerably below their actual content.

The significance of this coenzyme activity in nutrition has yet to be fully determined.

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SECTION II. FORMS AND CHEMISTRY

As Williams has stated:

> "An outstanding point of interest in the pantothenic acid investigation is the fact that so much of its structure was determined before it was obtained in pure form. In fact, the complete structure was known and the physiologically active substance was synthesized before pantothenic acid or its salts or other derivatives (excluding cleavage products) were obtained in condition to yield correct analyses. The most potent material obtained previous to synthesis was the calcium salt preparation described in 1929 (1)."

In this publication Williams stated that the free acid underwent acid or alkaline cleavage to yield beta-alanine and another hydrolysis product which appeared to be a dihydroxy valeric acid, linked through its carboxyl group to beta alanine through its amino group. In the month after Williams' publication Woolley, Waisman and Elvehjem (2) submitted a communication to the Journal pointing out that the properties of the chick antidermatitis factor and pantothenic acid were similar, since they were both heat- and alkali-labile hydroxy acids whose acetyl derivatives distill unchanged under the same conditions; and since the solubilities of the free acid and salts are alike for both substances. In this paper these workers postulated that the chick anti-dermatitis factor was a hydroxy acid in amide linkage with beta-alanine.

In 1940 Williams and Major (3) announced the synthesis of pantothenic acid by Doctors Stiller, Harris, Kerseztesy, Folkers and Finkelstein of the Merck Laboratories (4). The structure of the acid is shown in figure 21. The hydroxy acid part is known as pantoic acid.

Chemical and Physical Properties

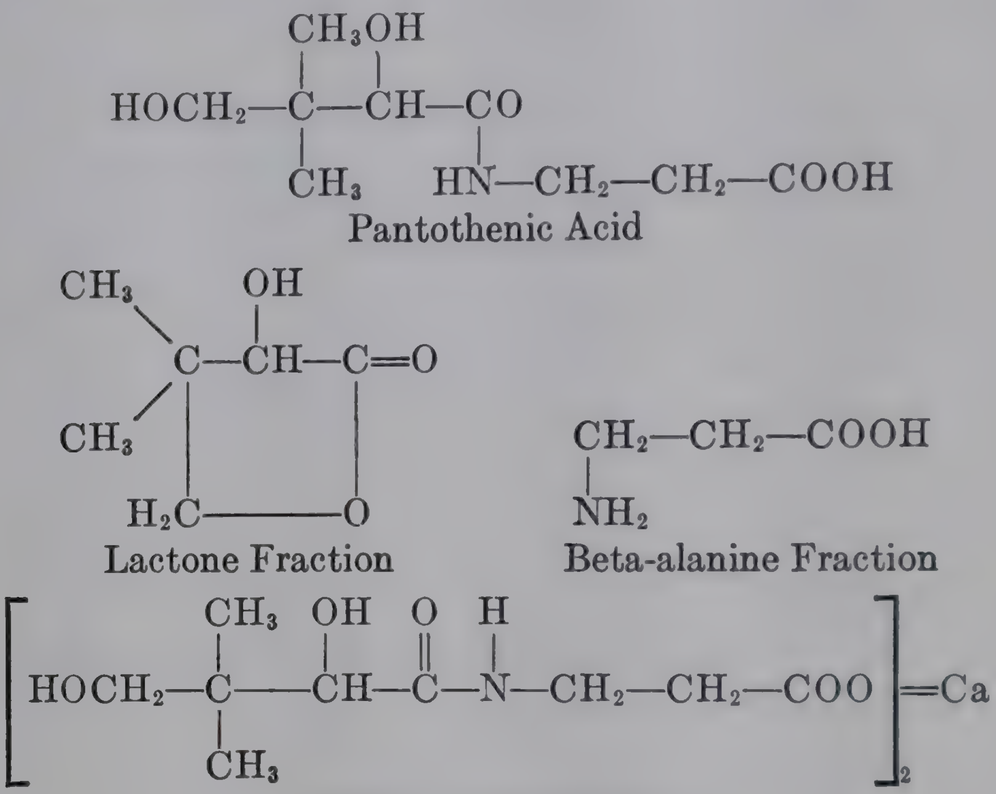
Chemical Name: Gamma di-hydroxy-beta, beta-dimethyl-butyro-beta′-alanide. Dextro-rotary calcium pantothenate has the empirical formula: $(C_9H_{16}N\,O_5)_2Ca$; molecular weight 476.5. Owing to the lability of the free acid the product is merchandised as the calcium salt which is in the form of a white odorless, crystalline powder with a slightly bitter taste.

One gram dissolves in approximately 6.9 cc. of water at 25° C. It is insoluble in alcohol.

The theoretical pH of a 5 per cent aqueous solution protected from CO_2 is 8.72. The calcium salt is stable to light and air. It is the dextro form of acid and salt that has the activity; calcium-1-pantothenate has less than 1 per cent of the activity of the dextro form when measured by bacterial assay.

Pantothenic Acid Analogs

A wide variety of pantothenic acid analogs have been prepared and tested for effect on the growth of micro-organisms and test animals. The requirement of the malarial parasite (Plasmodium lophurae) of pantothenic acid for growth (5) was one reason for such activity in making analogs in the hope of making a compound that would be antagonistic to pantothenic acid and act as an antimalarial agent.



$$HOCH_2-\overset{CH_3}{\underset{CH_3}{C}}-\overset{OH}{CH}-\underset{HN-CH_2-CH_2-COOH}{CO}$$

Pantothenic Acid

Lactone Fraction

$$\underset{NH_2}{CH_2}-CH_2-COOH$$

Beta-alanine Fraction

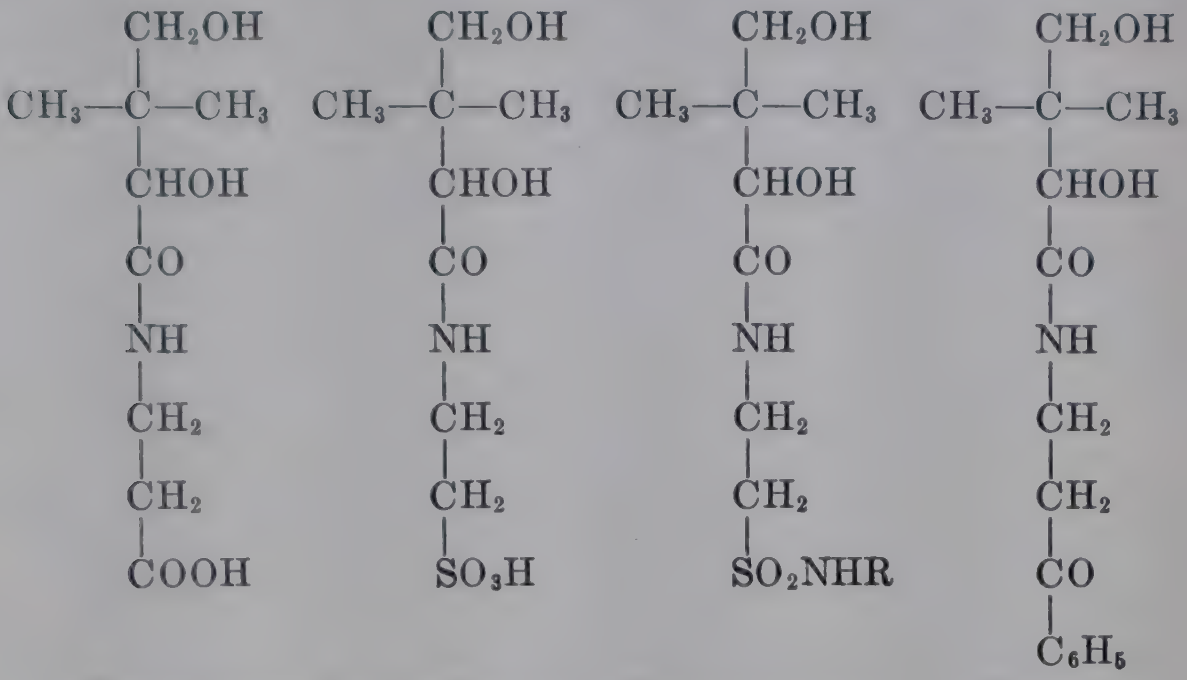
$$\left[HOCH_2-\overset{CH_3}{\underset{CH_3}{C}}-\overset{OH}{CH}-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-CH_2-CH_2-COO\right]_2=Ca$$

Calcium pantothenate

Fig. 21

Two compounds definitely antagonistic to pantothenic acid (pantoyltaurine (6) and phenylpantothenone (7) have had special study. Their structural relation to pantothenic acid is shown in figure 22.

Winterbottom et al (8) prepared 25 derivatives of pantoyl taurine and 5 derivatives of taurine. Pantoyl taurine and its amides proved to have high antibacterial and antiplasmodial activity in vitro. Against *S. hemolyticus* the amides were superior to pantoyl taurine itself and were similar to pantoyl taurine in that the d- forms were superior to the 1- forms. However, the amounts required to overcome pantothenic acid stimulation, were large. McIlwain (9) reported that pantoyl taurine was antagonistic to only those microorganisms requiring pantothenic acid for growth, but that it had to be present in amounts 500 to 1000 times the concentration of pantothenic acid necessary for growth. That fact probably accounts for failures to get antagonistic effects in certain animals (10, 11, 12), though McIlwain and Hawkins (13) have reported that when the ratio of pantoyl taurine to pantothenic acid was maintained above a certain range of blood level, it protected rats against 100,000 lethal doses of a strain of *S. hemolyticus*.



$$\begin{array}{c} \mathrm{CH_2OH} \\ | \\ \mathrm{CH_3{-}C{-}CH_3} \\ | \\ \mathrm{CHOH} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array} \quad \begin{array}{c} \mathrm{CH_2OH} \\ | \\ \mathrm{CH_3{-}C{-}CH_3} \\ | \\ \mathrm{CHOH} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{SO_3H} \end{array} \quad \begin{array}{c} \mathrm{CH_2OH} \\ | \\ \mathrm{CH_3{-}C{-}CH_3} \\ | \\ \mathrm{CHOH} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{SO_2NHR} \end{array} \quad \begin{array}{c} \mathrm{CH_2OH} \\ | \\ \mathrm{CH_3{-}C{-}CH_3} \\ | \\ \mathrm{CHOH} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{NH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{C_6H_5} \end{array}$$

Pantothenic Acid | Pantoyltaurine | Pantoyltaurine amide | Phenyl-pantothenone

FIG. 22. ANALOGS OF PANTOTHENIC ACID

The Cheldelin and Schink Study

Particular interest attaches to a report by Cheldelin and Schink (14) which appeared in 1947. In the prelude of this report they summarize previous studies as follows:

> "Analogs and derivatives of pantothenic acid have been prepared in a number of laboratories. The earlier ones resulted from studies on the structure of the vitamin (15), and from efforts to produce compounds with similar biological activity (6, 16, 17, 18, 19, 20). Following the preparation of pantoyl taurine (6, 21, 22), however, most syntheses have aimed at developing inhibitors related to pantothenic acid. . . . In general the most successful inhibitors have been those in which the pantoic acid moiety of the molecule is coupled to a suitable amino acid (21, 6, 23, 24), amino ketone (7), amino alcohol (25), or amine (24). Alterations in the pantoic acid moiety, on the other hand, have with but one exception (26) given rise to inactive or very slightly stimulatory substances, when tested on organisms requiring the preformed vitamin."

The products prepared and studied by Cheldelin and Schink and their effect on three types of organisms are shown in Table 44. They chose acetobacter suboxydans as one of these organisms because it utilizes pantoic acid as readily as the intact vitamin (27) and might be inhibited by compounds resembling this acid (1, 2, 3 of Table 44) as well as by their condensation products with beta alanine (4, 5, 6 of Table 44).

Study of the tabulated results will show that none of the pantoic analogs is able to counteract the growth-promoting effect of pantothenic acid on *L. arabinosus* which utilizes only the intact vitamin, or on Gebrüder Mayer yeast which can synthesize pantoic acid readily. In the case of *A. suboxydans*, however, where growth is dependent on pantoic acid there was competetive inhibition between antimetabolite and the growth factor. These antimetabolites would appear then to be able to prevent the coupling of beta-alanine to pantoic acid which normally occurs in *A. suboxydans* or yeast when the intact vitamin is not available. Yeast is less inhibited than *A. suboxydans* because pantoic acid is normally produced by yeast and it is known that organisms that can synthesize pantothenic acid are not in general affected by pantothenic acid analogs (7, 25).

TABLE 44

Effect of pantoic and pantothenic acid analogs on three organisms

(After Cheldelin and Schink (14) 1947)

	Analog/growth factor ratio @ 50% inhibition*			Gebruder Mayer yeast	
I. Pantoic acid analogs	Lactobacillus arabinosus 17-5	Acetobacter suboxydans			
	Pantothenic acid	Pantoic acid	Pantothenic acid	Beta-alanine	Pantothenic acid
1. $HOCH_2C(CH_3)_2CH_2COOH$	No inhibition	10	300	100,000	100,000
2 $CH_3C(CH_3)_2CHOHCOOH$	No inhibition	1000		25,000	No inhibition
3. $HOCH_2C(CH_3)(OH)CH_2COOH$	No inhibition	12,000	100,000	40,000	No inhibition
II. Pantothenic acid analogs					
4. $HOCH_2C(CH_3)_2CH_2CONHCH_2CH_2COOH$	0.02% act.	0.05% act.	0.03% act.	8% act.	3% act.
5. $CH_3C(CH_3)_2CHOHCONHCH_2CH_2COOH$	0.004% act.	0.02% act.	0.01% act.	90% act.	30% act.
6. $HOCH_2C(CH_3)(OH)CH_2CONHCH_2CH_2COOH$	No inhibition	0.01% act.		15% act.	3% act.
7. $CH_3C(CH_3)_2CHOHCONHCH_2CH_2SO_2OH$	No inhibition	800	2000	No inhibition	No inhibition
8. $HOCH_2C(CH_3)_2CHOHCONHCH_2CH_2SO_2OH$	1000	10% act.	10% act.	No inhibition	10,000
9. $HOCH_2C(CH_3)_2CHOHCONHCH_2CH_2CH_2CH_3$	12,500	0.04% act.	0.04% act	30,000	25,000

* Molar analog/growth factor ratios at 50% inhibition means where growth is equivalent to that produce by one-half of the growth factor present.

The effect of the 6 pantothenic acid analogs (4–9 of Table 44) is of interest. Numbers 4, 5, and 6 are beta alanides of the three pantoic acid analogs (1, 2, 3). Compound 7 was obtained by condensing taurine with pantoic analog number 2. Number 8 is pantoyl taurine and number 9 is N-pantoyl-n-butylamine. Note that, in contrast to the effect of the pantoic acid analogs, the pantothenic acid analogs produced from them (4, 5, 6) are of no value as growth inhibitors and have some growth stimulatory value to all the test organisms; that growth stimulation is greatest for yeast where compound 5 has virtually the same activity as beta alanine on a molecular basis, possibly because of hydrolysis by the yeast. The difference between the action of pantoyl taurine (8) and compound 7 is striking. Apparently the removal of the gamma hydroxy group changes pantoyl taurine into a compound completely inert for *L. arabinosus* and yeast. Conversely, while pantoyl taurine actually promotes the growth of *A. suboxydans* the desoxycompound is a good inhibitor perhaps because of the ability of the organism to hydrolyze pantoyl taurine to pantoic acid.

In their own discussion of the effects the authors note that with each organism studied, growth was influenced by the analogs in different ways. Some were inert, some showed vitamin activity and others competed with the growth factor presumably for attachment to the cell and probably at the surface of the enzyme. Changes in the beta-alanine structure rarely produce inert analogs and inert analogs appear to differ from the vitamin in the pantoic acid portion of the molecule, suggesting that the vitamin is normally attached through the pantoic acid group. Both hydroxy groups are important; removal of either one greatly reduces activity and changes shown in compound number 6 removed activity altogether. Further evidence of the attachment through the pantoic acid moiety is shown in the behavior of pantoyl taurine. While this inhibits growth produced by pantothenic acid in yeast it has no influence when beta-alanine is supplied (28). If both the growth factor and the analog were attached by means of the carboxyl or beta-amino group competition should be found but if we regard pantoic acid (produced by the cells) as being combined with the enzyme, beta-alanine would be free to couple without interference from pantoyl taurine.

With convincing proof that pantothenic acid occurs in the tissues as the coenzyme these studies are of special interest as throwing light on the possible attachment of the vitamin in this enzyme system.

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Section III. Evaluation of Pantothenic Acid in Human and Animal Nutrition

In spite of the fact that pantothenic acid has been found essential in the diet of all animals investigated and in various microorganisms, there is as yet no convincing proof of its essentiality for man. Evidence has been presented that it has an effect on achromatrichia in human beings but the evidence is conflicting as shown in Section I (p. 196). Evidence that it can function as a coenzyme in acetylation reactions may ultimately give it place in human nutrition, especially as the major part of the pantothenic acid in human tissues is in coenzyme form.

Gordon in 1942 (1) reviewed the evidence of its value clinically and cited certain data that have been reported. The first report of a clinical trial was by Spies et al (2). They noted a fall of up to 50 per cent in the blood level of pantothenate in patients with pellagra, beri-beri, and riboflavin deficiency. Injections of calcium pantothenate produced a rise in both pantothenic acid and riboflavin suggesting that in some way pantothenic acid is necessary for man and closely associated with riboflavin supply.

Gordon himself has reported (1) data on the urinary excretion of normal human subjects. In a series of assays on 40 different subjects he got a daily average of 3.52 mg. with a range from 1.14–6.36 mg. but none excreted less than 1.0 mg. He found in twelve cases of nutritional deficiency an average of 0.83 mg. with a range from 0.39 to 1.45 mg. From these data he drew the conclusion that 1.0 mg. each 24 hours was the dividing line between normal and nutritionally deficient subjects. Therapeutic administration of calcium pantothenate promptly restored normal excretion in the deficient subjects.

He also quotes Sebrell (3) as stating that 2.77 mg. of riboflavin is the requirement for normal adults who had a daily excretion of 700–1200 microgram of riboflavin daily. Gordon notes that if the same ratio between excretion and requirement holds, the human daily requirement for pantothenate should be 9–11 mg. daily. This, however is obviously a speculation and not demonstrable until experimental pantothenic acid deficiency is actually produced in human subjects.

Cheldelin and Williams (4) have estimated that the average American diet supplies 4.5 mg. of pantothenic acid per 2500 calories. If Gordon's figure is confirmed this would indicate inadequacy to be common.

In their study of its distribution in human tissues Taylor, Pollack and Williams (5) found it present in all tissues examined ranging from 2.0 to 45 micrograms per gram of tissue; highest content in the liver, adrenals, heart and kidneys.

Its place in human nutrition has yet to be established and must await the demonstration of a syndrome in the human subject definitely producible by pantothenate deficiency and preventible and curative by pantothenic acid.

Pantothenic Acid and Domestic Animals

Cattle and Sheep: After attainment of two years of age, rumen synthesis of pantothenic acid appears adequate to meet the need of cattle and sheep. Pearson and Darnell (6) have reported on the distribution of pantothenic acid in the colostrum and milk of cows and ewes. In both animals the content of the colostrum is significantly lower than that of the milk. Concentration in the milk increased on the second day and by the third day had reached the normal milk content.

Swine: Pantothenic acid deficiency in swine is manifested by a wobbly gait (goose stepping), myelin sheath degeneration of nerves, scurvy, thin hair, brownish secretion around the eyes and in severe cases bloody feces. The effect of pantothenic acid and pyridoxine on the nervous system of swine has been discussed in the previous pages. The allowances recommended by the Committee on Animal Nutrition of the National Research Council (7) are 10 mg. daily for 50 lb. pigs; 18.5 mg. per day for 100 lb. pigs. No data on adult animals are given.

Poultry: In Section I the effect of pantothenic acid in the chick was detailed. The Committee on Animal Nutrition list the following: In young chicks pantothenic acid deficiency retards growth and ragged feathers develop. Within 12 to 14 days a pellagra-like symptom develops. The eyelids become granular and stick together because of a viscous exudate. Crusty scabs appear at the corners of the mouth and around the vent. Dermatitis of the feet is also observed. Liver damage and spinal cord changes show on postmortem examination. Such lesions have not been observed in mature fowls but pantothenic acid deficiency does lower the hatchability of eggs (9).

The Committee's recomended allowances (8) are 5 mg. per pound of feed for growing chicks; 7.0 mg. per pound of feed for laying and breeding hens.

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CHAPTER XII. INOSITOL

SECTION I. FUNCTIONS OF INOSITOL

The Bios Postulate

While, as stated in the previous chapter, pantothenic acid perhaps most nearly resembles the yeast growth stimulant designated by Wildier (1) in 1901 as "bios", it was not the first yeast growth stimulant isolated.

In 1924 Eddy, Kerr and Williams (2) reported the isolation of a crystalline substance with an empirical formula of $C_5H_{11}NO_3$, a compound closely resembling an amino acid reported by Mueller (3) to which he assigned the formula $C_5H_{11}NSO_2$. R. J. Williams has suggested that it would have been possible for this product to have been over 99 per cent pure and still be contaminated with enough pantothenic acid to account for its yeast stimulation effect (5 mcg/cc. of culture medium). The chemical structure of this compound was never determined but possibly it was a stimulatory amino acid; since yeast does respond to the stimulation of the amino acid beta alanine and perhaps to others. The product appeared chemically pure. It has a melting point of 223°C, an index of refraction of 1.52–1.53 and a molecular weight of 133. Experiments suggested that it was a heterocyclic nitrogen carbon ring complex with a carboxyl group attached.

About this time evidence had accumulated to suggest that bios was multiple in nature (4). This was supported by the isolation of one such bios from tea leaves by Eastcott (5) in 1928 under the name of Bios I. This product proved to be the cyclic sugar known as inositol. Eastcott's discovery established inositol as a nutrilite but Woolley's (6) report that it prevented alopecia in mice and Pavcek and Baum's (7) report that it corrected a loss of hair around the eyes of rats (spectacle eye) first gave it claim for a place in animal nutrition and for a possible vitamin status.

Claim of Vitamin Status

For a nutrilite[1] to claim vitamin potency it must act on animals, producing specific effect by deficiency and curative action by therapy. In 1944 Woolley (8) reviewed the nutritional significance of inositol and its justification for classification among the water soluble vitamins. In this review he cites the following effects of inositol deficiency:

---

[1] In 1928 R. J. Williams suggested the name "nutrilite" for factors with "bios" activity for these reasons:

> "The term vitamin carries unfortunate etymological implications and its use should not be unduly extended. The term "nutrilite" on the other hand, is unobjectionable from this standpoint since it implies only an importance in nutrition and nothing as to the chemical nature of the agent."

In mice: Retarded growth and alopecia.

In rats: Retarded growth, general alopecia, spectacled eye, fatty livers and a relation to lactation failure.

In cotton rats: and guinea pigs: Growth retardation.

In hamsters: Some growth retardation and effect on reproduction.

In chicks: Growth retardation, encephalomalacia and exudative diathesis.

Inositol as a Lipotropic Factor

Special interest attaches to evidence that inositol might be of value in human nutrition as a preventive of fatty livers.

The normal liver contains about 4 or 5 per cent fat. In man and in animals a variety of conditions can lead to an abnormal increase in liver fat definitely affecting liver function and leading to cirrhosis. There are two general causes of fatty livers: 1) by increase in the rate at which fat is supplied to the liver or 2) by a decrease in the rate at which the liver is able to dispose of the fat it receives.

An increased supply of fat to the liver can be produced by a) a high fat diet; b) by starvation and accelerated transport from depots; c) by stimulation with the ketogenic fraction of the anterior lobe of the pituitary; d) a possible increased liponeogenesis from carbohydrate and protein supplying fat faster than the liver can handle it.

A delay in removal of fat from the liver by oxidation or transport may be produced by a) poisoning (e.g. carbon tetrachloride, yellow phosphorus); b) a high cholesterol diet; c) in dogs, by depancreatization plus insulin; d) choline or other lipotropic factor deficiency.

McHenry and Patterson (9) reviewed in 1944 some of the "lipotropic" factors that help remove fat from the liver with special attention to choline, lipocaic, and inositol. Folch and Woolley (10) had shown that inositol was a component of a mammalian phospholipid, lipositol or liposterol, and that it could act as a lipotropic factor under special circumstances.

Gavin and McHenry (11) have described the production of a fatty liver in rats by the following procedure: for 3 weeks, rats on a fat-free, 10 per cent casein diet were deprived of choline and all water-soluble vitamins. Following this period, on the fourth week the diet was supplemented with various test materials and the livers analysed for fat. With no supplement the liver fat was normal, 3-4 per cent. Thiamine supplement increased fat content to about 8 per cent and choline prevented this increase, but if the supplement included riboflavin, pantothenic acid, pyridoxine, and niacin in addition to thiamine and choline, a 10 per cent fatty liver developed in spite of the choline. Again if to the supplements the vitamin biotin was added, the liver fat increased to 25 per cent without choline and 18 per cent with choline. According to McHenry and Gavin these so-called "biotin fatty livers" were completely prevented by giving inositol in addition to choline.

To summarize, these biotin fatty livers were said to be characterized by a high content of cholesterol, and inositol was reported to be more effective than choline in reducing the level of cholesterol esters in the liver lipids and also in those resulting from feeding of a high cholesterol diet.

Further investigation of this phenomenon was reported by Handler (12) in 1946. With a low fat diet plus a basal vitamin B supplement, fatty livers were obtained which could be prevented by choline and to a lesser extent by inositol. Administration of liver extract (containing biotin) caused fatty livers only partially prevented by choline, somewhat better by inositol, and completely by a combination of choline and inositol. The effect of the liver extract could be almost exactly duplicated by a combination of folic acid and biotin. With a high fat diet fatty livers were produced in the presence of choline and inositol without the addition of liver extract, folic acid or biotin but were prevented by a combination of choline and inositol. Handler, however, reached the conclusion that fatty livers due to choline deficiency are not found in animals which are for any reason losing weight. The extent of the accumulation of liver fat in choline deficiency is roughly proportional to the food consumption and the growth rate; nor is there real justification for the term "biotin fatty liver."

The idea of the biotin fatty liver theory has also been challenged by Beveridge and Lucas (13) who reported inositol not more but actually less effective in reducing cholesterol esters and that choline was at least as effective in reducing bound cholesterol and that no difference exists between fatty livers caused by biotin and by a high fat diet. Best et al (14) have also reported failure to find any support for the conclusion that biotin produces a selective deposition of cholosterol esters in the liver, that inositol has a specific effect on cholesterol esters, or that the fatty liver produced after administration of biotin is peculiarly resistant to choline.

The significance of the synergistic lipotropic action of a choline-inositol combination remains to be cleared up, and also the possible relation of biotin to inositol. The possibility of use of inositol for the correction of fatty livers in human subjects remains a matter for further study.

Inositol and "Spectacled Eye" in Rats

As already noted Pavcek and Baum (7) reported that a phase of alopecia in rats resulting in a denuded area around the eyes of the animals which gave them a spectacled appearance was a specific inositol deficiency effect. In the same year, however, Nielsen and Elvehjem (15) reported it to be a biotin deficiency effect.

Lindley and Cunha (16) have reported that when pigs are fed a highly purified ration plus sulfaphthallidine they develop a syndrome similar to that seen in egg white-induced biotin deficiency and that this disease could be combatted by either biotin or inositol.

Spitzer and Phillips (17) have stated that when the protein of an otherwise synthetic diet for rats is supplied by soy bean meal, an alopecia identical in type to that seen in inositol deficiency in mice develops. This condition is also preventable by either inositol or biotin. These observations indicate further need for study to clarify the interchangeability of the two vitamins.

Encephalomalacia and Exudative Diathesis

In the chapter on vitamin E functions (See p. 65) is given a description of the encephalomalacia and exudative diathesis in chicks resulting from vitamin E deficiency. Dam (18) has reported that these effects can be prevented by addition of inositol to the diet. It has also been observed that tocopherol-deficient chicks need something they get in soy bean meal in addition to tocopherol itself and since Woolley (10, 19) has shown that the oil is rich in a phosphatide called liposterol which contains inositol, it is possible that inositol is the factor in soy bean oil to which the chick responds.

Inositol and Reproduction

Another evidence of inositol's similarity to tocopherol in action is found in a report of its effect on the reproduction of the hamster. In 1943 Cooperman et al (20) reported that hamsters required biotin, paba or inositol or both. When biotin was given and paba and inositol witheld, the animals did poorly and one third to one half died within four weeks. Hamilton and Hogan (21) could not confirm the need for biotin, inositol or paba for growth but did find a need for vitamin E and K. They found inositol necessary, like vitamin E in rats, for the delivery of living young. On inositol-deficient diets several femals died during or shortly after parturition with one or more decomposed embryos in the uterus. Of 25 litters 20 were still-births.

Inositol and Muscular Dystrophy

Still another resemblance of inositol to vitamin E appears in its possible relation to the prevention of fibrositis. Sternberg (22) in 1941 reported successful use of vitamin E in 30 cases of human fibrositis. In 1945 Milherat and Bartels (23) (See p. 78) reported that when inositol and tocopherol were fed together in a combination such as the mono-ether of inositol and tocopherol, the combination was more effective than tocepherol alone.

Inositol and Lactation

In 1938 Nakahara et al (24) postulated the existence of two vitamins L_1 and L_2 extracted from beef liver and bakers' yeast necessary to lactation in the rat, and that they functioned in the maturation of the lactation tissues. Several groups of American investigators (25, 26, 27), have reported paba and inositol to be lactation factors for the rat. Folley, Henry and Kon (28) failed to confirm the work of the Japanese investigators but their diet did contain paba and inositol, and these may be the vitamins the Japanese called L_1 and L_2. (See p. 351).

Pharmaceutical Effect of Inositol

Bly et al (29) studied the effect of pantothenic acid and inositol on motility, digestion and absorption in the upper region of the intestinal tract of the dog. Inositol given orally in amounts as great as 500 mg. per day temporarily increased motility but decreased digestion and absorption, and also acted more like a cathartic.

It may be that large amounts of caffeine in coffee may create an iositol deficiency. It has been reported that when roasted coffee (not the decaffeinated kind) was added with niacin to the basal diet of dogs, a paralysis resulted that was curable by inositol. An eye condition corrected by biotin also occurred. The suggestion is that the inclusion of caffeine in the diet can create a biotin and inositol deficiency.

Inositol as a Nutrilite

As noted, Eastcott (5) was the first to establish bios activity in inositol. R. J. Williams has reviewed its value in this role and notes that when added by itself to a synthetic sugar-salt medium its effect is practically nil; it becomes a limiting factor only when other nutrilites are supplied. Also when yeasts grow in the absence of inositol, they actually synthesize it.

Janssens (30) found it indispensable for Wildier's yeast and for old process yeast. Williams et al (31) found it increasingly important for the following yeasts in the order of their naming; Fleischman bakers' yeast, Gebrüder Mayer yeast and old process yeast.

Lochhead and Landerkin (32) found 5 strains representing 3 species of zygosaccharomyces required it for optimum growth, *zygosaccharomyces necturophetus* in particular. Buston and Pramanik (33) report it an essential nutrilite for the fungus *nematospora gossypii* which is parasitic on cotton bolls.

It is known to be formed in the intestinal tract by certain bacteria (35). Thompson (34) has listed the following bacteria that synthesize inositol: *Aerobacter fluorescens* (1.4–1.6 mg/gram dry cells); *Serratia marceseus*

(1.6 mg/gm); *Pseudomonas fluorescens* (1.7 mg/gm); *Proteus vulgaris* (1.0 mg/gm); *Clostridium butylicum* (0.87 mg/gm).

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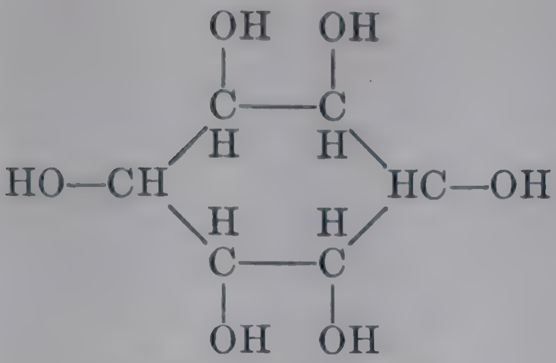
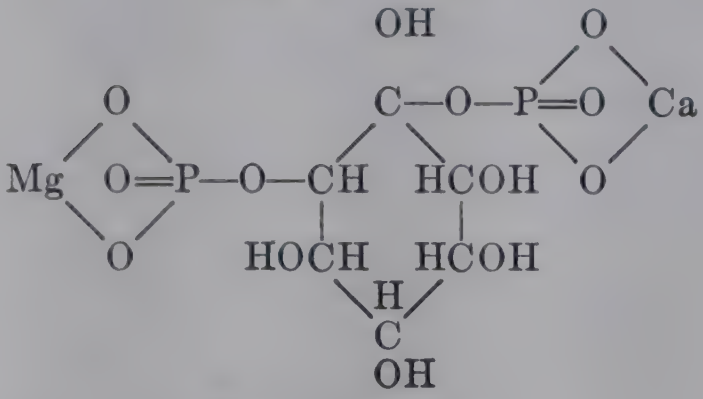


Meso or i-inositol



Phytin Structure

Phosphorylated Inositol with Magnesium and Calcium

(N. B. the diagram shows attachment of two of the six phosphoric acids which make up the complete compound.)

FIG. 23. FORMS OF INOSITOL

TABLE 45

Activity of forms of inositol

(After B. C. J. G. Knight (2) 1945)

Compounds	Curative effect on mice. Dose activity in mcg. per 100 gms. of ration	Yeast growth activity expressed
		per cent
Meso-inositol	100	100
Mytilitol	200	10
Inositol mono-phosphate		5
Inositol tetra-phosphate		2
Phytin	100	1
Soy bean cephalin	2000	1

SECTION II. FORMS AND CHEMISTRY.

Like niacin, inositol was known many years before there was suggestion of its being a nutrilite or a vitamin. It was one of the non-nitrogenous extractives of muscle.

Inositol nutrilite as isolated by Eastcott (1) is the meso- or i-inositol shown in figure 23. It occurs in bound form as Phytin, the calcium magnesium salt of inositol phosphoric acid, also in soy bean oil as liposterol or lipositol. Liposterol has been separated from the cephalin fraction of animal brains and from the spinal cord. In Table 45 is shown a comparison of forms of inositol and their relative potencies for mice and yeast as reported by Knight (2). Fisher (3) has reviewed the chemistry of inositol in detail.

Chemical and Physical Properties

It is an alcohol, not an aldehyde.

It crystallizes in large rhombic crystals melting at 225° C.

Soluble in water (1 gm. in 7.5 cc.); insoluble in strong alcohol and in ether.

It has the formula of a hexa-hydroxy-cycle-hexane.

There are eight possible isomers, only one of which, the optically inactive i-inositol or meso-inositol is biologically active.

It crystallizes from acetic acid or water above 50° C as the water-free compound (M.P. 225–226 °C.); at below 50° C as the dihydrate (M.P. 215–216° C.).

It was found non-toxic to mice when fed in amounts as much as 10 mg/100 gm. of ration; to rats at 20 mg/100 grams of ration.

Potency is expressed in weight and the product has been synthesized.

Anti-inositol

A mixture of isomeric hexachlorocyclohexanes is used in commerce as the insecticide "666" and is claimed by Slade (4) to owe its activity almost wholly to the gamma isomer. Studies by Kirkwood and Phillips (5) showed that the gamma isomer markedly inhibited the growth of yeast and that this inhibition was progressively but not completely reversed by the addition of 1–6 mcg of i-inositol. This discovery supports Slade's claim that the gamma-hexachlorocyclohexane exerts its insecticidal action by interfering with the inositol metabolism of the insect.

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SECTION III. EVALUATION OF INOSITOL IN HUMAN AND ANIMAL NUTRITION

As in the case of pyridoxine and pantothenic acid the Council on Pharmacy and Chemistry of the American Medical Association has held that:

> "Because no symptom complex in man has been defined attributable to a deficiency of inositol, no therapeutic claims have as yet been recognized."

Nutrition Reviews (1) puts the situation in 1946 as follows:

> "The position of inositol as a dietary essential now appears to be assured but little is known concerning its value in human nutrition. A report has appeared which indicates that under certain conditions, inositol is a lipotropic factor in man, that is, it is effective in preventing and curing fatty livers."

To this might have been added that if the work of Milhorat and Bartels (2) (see p 212) is confirmed it may prove of value in the treatment of certain types of muscular dystrophy such as fibrositis.

Inositol in the Nutrition of Animals

In discussing the vitamin needs of cattle, sheep, swine, and poultry the Committee on Animal Nutrition of the National Research Council (3) makes no mention of inositol as a dietary essential for any of these animals. That it is of significance in the diet of chicks and pigs has already been noted. (See pp. 210 and 212.)

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CHAPTER XIII. PABA (PARA-AMINO-BENZOIC ACID)

SECTION I. FUNCTIONS OF PABA

The compound para-amino-benzoic acid, (Paba for short), was first synthesized by Fischer (1) in 1863. However, its claim to be a vitamin dates only from 1940-41.

In 1941 Woods (2) and Fildes (3) postulated that an antisulfonamide present in yeast was paba and that paba is an "*essential metabolite*" for the growth of bacteria but is normally synthesized in sufficient quantity by many strains of bacteria.

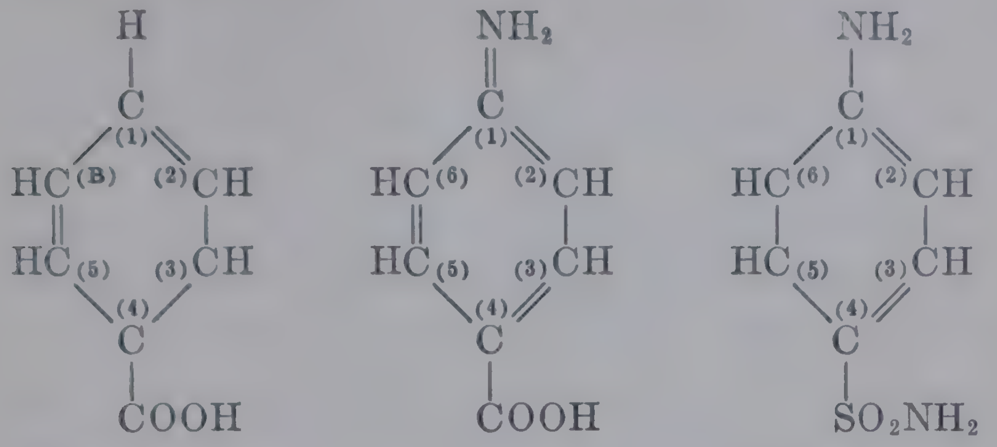
> "By definition, an "essential metabolite" is a substance that takes an essential part in a chain of syntheses necessary for bacterial growth, in contradistinction to a "growth factor" which must be supplied in the nutrients as an essential metabolite that the cell cannot synthesize." (44).

Support for this view of paba as an essential metabolite was given by Landy et al (5) who reported that representative cultures of many different bacterial genera were grown on media of known composition and free of paba. All of the organisms synthesized paba in readily measureable amounts, showing that it is an essential metabolite.

Woods and Fildes also advanced a hypothesis to explain the antagonism of paba and the sulfonamides based on the structural similarity of the two compounds (See figure 24). Wooley (6) has put this theory as follows:

> "Woods observed that the bacteriostatic action of the sulfonamides was reversed completely, competitively by paba, the structural analog of sulfanilamide. The chemical relationship here was that sulfanilamide is paba with a sulfonamide group instead of the carboxyl group. The hypothesis was advanced that the sulfonamides owed their action in inhibiting growth of bacteria to their competition with paba in an essential metabolic reaction." . . . After the discovery of the reversal of sulfonamide bacteriostasis by paba, the hypothesis was advanced that the sulfonamides competed with paba for a place on the receptor portion of an enzyme. The enzyme was supposed to require paba as a coenzyme, and the introduction of a sulfonamide was supposed to result in a firm combination of the latter with that portion of the enzyme to which paba would normally be attached. The resultant foiling of the enzyme was said to be overcome by an increase in concentration of paba which would shift the equilibrium in favor of the paba-enzyme combination. Despite the fact that there has been no direct experimental proof of an enzyme system involving paba, this attractive hypothesis has continued to flourish with slight opposition."

In 1941 Ansbacher (7) claimed paba had chromotrichial effect on rats and a growth effect on chicks. The latter claim has been supported by other investigators (8, 9). These claims suggest that paba may be granted a place in the vitamin classification.



Benzoic Acid Paba Sulfanilamide

(para-amino-benzoic acid)

FIG. 24. RELATION OF PABA TO BENZOIC ACID AND SULFANILAMIDE

Of this claim Ansbacher (4) has commented as follows:

> "By definition, a vitamin B-Complex factor is:
> a) a natural constituent of yeast, liver and/or cereals.
> b) is water-soluble.
> c) a growth substance for bacteria, yeasts, fungi and/or molds.
> d) a coenzyme or activator of enzymatic processes.
> e) physiologically active in small amounts.
> f) a substance that causes a deficiency disease when lacking in the diet.
>
> The data presented in the preceding chapter (his review (4)) seem to justify the conclusion that paba is one of the vitamin B-complex factors; it occurs in nature as universally and side by side with most B vitamins; its role as a phenolase activator has been suggested; it has been claimed to be clinically effective in amounts comparable to those employed in choline and inositol therapy; deficiency symptoms resulting from its absence from the diet have been described."

Evidence of Chromotrichial Potency

In 1941 Ansbacher (7) reported that:

> "Experiments conducted in this Institute (Squibb Institute for Medical Research) indicate that p-amino benzoic acid, considered to be an essential metabolite for bacteria by Fildes (10) is a vitamin, namely a chromotrichial factor for the rat and a growth promoting factor for the chick."

A similar color effect on mice was reported by Martin and Ansbacher (11) in the same year but was challenged by three other groups of investigators (12, 13, 14). Martin (15) in 1942 attempted to reconcile these opposing findings. In brief he drew the conclusion that paba is chromotrichial only as it alters intestinal flora, thus favorably influencing the bacterial synthesis of folic acid which is the actual chromotrichial agent. Wright and Welch (16) advanced a similar explanation of paba's chromotrichial effect: that it is a result of its stimulation of intestinal bacterial syntheses whose products may in turn improve the utilization of pantothenic acid.

If this explanation is correct, it is paba's action as an essential metabolite rather than its direct action on melanin formation that accounts for its color restoration action.

To date four vitamins have been cited which affect color of hair and its restoration to natural color, namely, pantothenic acid, paba, biotin, and folic acid. It is also now known that paba forms a part of the folic acid molecule.

There has also been conflict over the claim that paba can influence human hair color. Sieve (16) and Banay (17) have supported the claim but their findings lack satisfactory confirmation.

The Chick Growth-promotion Effect Claim

As already noted it was Ansbacher in 1941 (7) who first reported a growth-promoting effect on chicks. He stated:

> "Chicks reared on the heated vitamin K deficient diet described (18) were found to show only a small gain (less than 100 gms) in weight and to die within about a month even when ample amounts of calcium pantothenate and of vitamin K active 2-methyl-1,4-naphthoquinone were fed. However the addition of 300 mcg of paba per gram of ration resulted in better growth and longer survival times."

Hammond et al (8) reported that the chick requires paba for keel bone formation when reared on a heated diet deficient in phosphorus. Wisconsin investigators (19) were at first unable to demonstrate that the simple aromatic amine is needed by birds maintained on a heated diet. However, (9) they later reported further studies and discussed a possible mechanism of action. Apparently 5 mg. of paba per 100 gm. of ration had no effect, but 7.5–15 mg. gave noticeable responses in both growth and feather formation in chicks receiving purified rations known to be low in "unknown" vitamins and considered adequate in all other respects. It was also noted that paba stimulated production of folic acid and growth of micro-organisms in the chick intestine. They therefore advanced the view that paba is not a specific growth factor for chicks but may act indirectly by stimulating the intestinal bacterial to produce vitamins needed by the chick.

Again its function as an essential metabolite is invoked to explain the chick growth promotion.

Effect of Paba on Lactation

In 1941 Sure (20) reported that paba might be an essential dietary factor for rat lactation, perhaps identical with a part of a lactation factor he had previously characterized as Bx. Later (21) he reported that rice bran extract and a liver extract supplied a factor, the presence of which resulted in 90 per cent successful lactation. Negative tests of the ash of these sources proved it to be organic in nature. He suggested that paba and perhaps inositol might be components of his Bx.

Climenko and McChesney (22) however, while affirming that inositol is needed for lactation by the rat, claimed that paba did not increase lactation directly when given to the rat but when given to rats receiving inositol it slightly decreased the mortality rate of the new born.

To this Sure (23) in 1943 produced further evidence to show that paba did have a remarkably favorable influence on the lactation of albino rats while inositol had a pronounced injurious effect that could be counteracted by paba.

In 1938 Japanese investigators (24) postualated the existence of two lactation factors which they designated as L_1 and L_2; L_1 from beef liver and L_2 from bakers' yeast. At one time it was suggested that paba might be L_1 and inositol, L_2. However the Japanese workers later gave evidence to disprove that L_1 was paba and that it was actually anthranilic acid.

Just What is the Function of Paba?

It would be satisfying at this point to give a concise statement of how paba functions, what role it plays in an enzyme system or in direct action. Unfortunately knowledge has not progressed to a point to make this possible. Various theories of action have been advanced and are worth study (4, 25, 26, 27). The situation has been well summarized by Green (28) from whom the following quotation is taken:

> "The discovery of Woods that the antibacterial action of the sulfonamides could be explained in terms of the resemblance of the sulfonamides to paba was an important milestone in our understanding of the mechanism of action of drugs. It became at once clear that some enzymic process was at the bottom of the chemotherapeutic action of the sulfonamides. *There is still no clue as to the nature of this enzymic process but there can be little doubt that the process is enzymic.* Paba is a naturally occurring substance in yeast and animal tissues. Many bacteria and molds are unable to grow unless it is present in the medium. *The trace concentrations in which it must be present for optimum growth exclude all but a catalytic role.* In fact, paba has been shown to exist in yeast largely as a polypeptide, which may well be its active catalytic form in the intact cell.
>
> One theory of sulfonamide action assumes that the sulfa drugs displace paba from its combination with specific proteins and thereby inactivate enzymes important in the growth of certain microorganisms. In other words the natural substance, viz., paba, competes with the drugs for a protein partner with which it forms the enzyme complex. The degree of inhibition is determined by the relative concentrations of paba and drug and by their relative affinities for the protein partner. . . . The interpretation in terms of enzymes of the mode of action of sulfonamides is by no means in

general currency. The so called "essential metabolite" theory of Fildes has gathered many adherents and is generally accepted among workers in the field of chemotherapy. This theory assumes that paba is an essential metabolite for the growth of certain organisms rather than a part of an essential enzyme system.

A metabolite is usually defined as a substance which undergoes chemical transformation; and the term is usually applied to substances like amino acids, fatty acids etc., which are present in considerable concentration, and which are degraded or converted into more complex substances by enzyme systems. Since the amount of paba present in micro-organisms is scarcely detectable by the most delicate chemical methods, paba can hardly be classified as a *typical* metabolite. Quite clearly the term metabolite as applied to paba must imply that it is involved in metabolism. Furthermore, since traces of essential substances are known to participate in metabolism only in the capacity of catalysts, the "essential metabolite" theory boils down to a disguised enzyme theory. In the present state of ignorance there is some merit in talking of paba as an essential metabolite until such time as its place in the enzymic role is clarified. But when the essential metabolite theory is seriously proposed as an alternative to the enzyme theory, it becomes important to recognise what the concept of essential metabolite really means. As applied to paba, essential metabolite is just a term of caution to indicate by implication a catalytic role, without stating it in so many words."

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TABLE 46

Distribution of free and bound paba in various sources

(After Ansbacher (4) 1944)

	Total paba	Unbound paba
	p.p.m.	*p.p.m.*
I. *Animal tissues*		
Blood (rat)	0.27	0.06
Beef liver	2.5	0.2
Pork	0.8	0.3
Muscle (beef)	0.6	0.3
Brain (rat)	0.7	0.14
Heart (rat)	1.35	0.15
Kidney (rat)	1.8	0.13
II. *Dairy products*		
Milk	0.4	0.07
Egg yolk		0.8
Egg albumen		0.06
III. *B-complex sources*		
Yeast (brewers)	9–59	6–61
Rice bran	16	2
Whole wheat	0.8	0.25
Wheat germ	1.8	0.5
Molasses	0.3	0.01–0.2
IV. *Enzymes*		
Catalase (beef liver)	19	4.3
Phosphorylase	13	2
Rennin	19	1.0
Urease	21	1.9

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Section II. Forms and Chemistry of Paba

As shown in figure 24, paba or para-amino-benzoic acid is a simple aromatic amine with the amine group in the para position. It occurs in nature in both free and bound form (see Table 46). Bound paba may be in combination with carriers such as proteins or in combination with other compounds such as polypeptides and amino acids, or as conjugated paba, e.g. acetylated paba. It has been found in assaying paba sources that in general the total paba is usually much in excess of the free paba.

With the elucidation of the structure of the folic acids it was of interest to note that paba forms a part of the molecule of at least three of its forms.

Properties of Paba

Paba occurs as a colorless amorphous powder or in needle shaped crystals; melting point 186–187°C. Slightly soluble in water and moderately soluble in alcohol. Chemical formula (See fig. 24) $C_7H_7NO_2$; mol. Wt. 137.

SECTION III. EVALUATION OF PABA IN HUMAN AND ANIMAL NUTRITION

There are as yet no allowable claims for clinical use of paba. However, experiments with the product in the treatment of rickettsial disease suggest a possible important use in medicine.

For some reason sulfa drugs, penicillin and other forms of chemotherapy have proven ineffective in virus and rickettsial diseases. The agents are obligate, intracellular parasites and the environment of the cell appears to prevent the effectiveness of the drugs.

In 1944 Grief et al (1) pointed out that rickettsial growths are often associated with a low rate of metabolic activity in the host cells. This suggested that production of small changes in the metabolic state of the host cells might create an environment unfavorable to the growth and multiplication of the invading agents. On this basis they tested the effect of paba as a therapeutic agent against murine typhus in mice. A dog chow containing 3 per cent of paba provided striking protection for the infected mice.

These observations supported earlier observations by Snyder et al (2) and the report of the U.S.A Typhus Commission in Egypt on the beneficial effect of treating louse-borne typhus with paba.

In 1945 Anigstein and Bader (3) also tested the effect of paba on guinea pigs infected with Rocky Mountain Spotted fever and obtained favorable results which indicated that when paba was given before or shortly after infection has set in it could prevent the appearance of the clinical manifestations of spotted fever rickettsiae. This period is then followed by immune phenomena by which the guinea pig acquires active resistance against the infection.

Evidence from human experiments has been accumulating to confirm the value of paba in the treatment of spotted fever (4, 5, 6) (10) (11) (12) and in the treatment of Tsutsugamushi disease or scrub typhus (7).

The American Medical Association Journal (8) commented editorially on the theory of action and support the contention of Grief (1) and note that it is in line with Zinsser's (9) earlier experience, viz., that under conditions of high metabolic activity as measured by oxygen consumption, multiplication of intracellular ricketssiae did not occur in Maitland's cultures.

These findings should give paba a place in the physicians armamentarium but we have no data on the requirement for maintenance of normal human nutrition.

The great majority of the studies of paba behavior have been devoted to its use in the attempt to explain the chemotherapy of the sulfa drugs.

The situation with domestic animals and paba requirements is similar to the situation with human subjects. Its indirect effect through stimulation of intestinal bacteria to the production of factors the animals need has already been discussed in connection with chick growth and lactation effects in Section I. There are no definite suggestions as to amounts to be fed in building satisfactory animal diets. When paba is included in commercial vitamin mixtures the amount present per unit must be stated but accompanied with the statement "human requirement not known".

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CHAPTER XIV. BIOTIN

SECTION I. FUNCTIONS OF BIOTIN

Biotin owes its name to Kögl's search for a "bios" factor. In the search for this yeast growth promoting factor first postulated by Wildiers (1) it was demonstrated that bios was multiple in nature. Bios I, precipitable by lead acetate, was identified by Eastcott (2) as meso-inositol. Bios IIa was identified by Williams et al (3) as pantothenic acid. Bios IIb (adsorbable on charcoal) attracted the attention of Kögl and in 1935 he (4) announced the isolation from egg yolk of minute amounts of a crystalline compound possessing the greater part of the yeast growth activity of the Bios IIb fraction. This compound he called "biotin".

In 1933 Allison, Hoover and Burk (5) described growth-promoting and respiration-promoting effects of extracts from various sources on Rhizobium trifolii, a legume nodule organism. They called the active agent "coenzyme R".

In 1927 Boas (6) described an injury produced in rats by feeding raw egg white, also the occurrence in various foodstuffs of a factor which prevented and cured this injury. Gyorgy and coworkers (7) tracked down this protective factor and called it "vitamin H" after the German "Haut" for skin.

The combination of the efforts of Gyorgy and duVigneaud et al (8) demonstrated that Kögl's biotin, coenzyme R, and vitamin H were identities. Melville (9) has discussed in detail the combined work that elucidated the chemical nature of biotin which is discussed in Section II.

Egg White Injury

Boas (6) described the rat syndrome produced by feeding raw egg white as follows:

> "The rats grew well and are usually in good health for from 2 to 3 weeks. Then red scaly patches appear at the corners of the mouth, the coat becomes rough and sticky and the long hair falls out. The fur on the abdomen shows at first a characteristic ribbed appearance, followed by the development of bald areas. Meanwhile the red patches spread to other parts of the body and the picture is one of eczematous dermatitis. There are even skin hemorrhages in severe cases. The region around the mouth is always more severely affected though there is often such marked blepharitis that the eyes are closed. The loss of hair is often extensive. In a few cases edema of the feet has been seen but does not usually occur. These rats always have a distinctive, somewhat musty smell, probably due to some constituent of the urine. The body weight remains stationary for a week or two but falls slowly during the second stage of the disease. This is reached about two to three weeks after the development of the first signs of deficiency. To the dermatitis, symptoms of nervous upset are now added. There is pronounced spasticity of the limbs, particularly of the hind

> legs, and the back is arched. The rat assumes in many cases a kangaroo-like posture. . . .Some of the rats do not show marked spasticity but assume a crouching attitude, and display a curious swimming movement with the front paws. Death, which occurs in the final phase, is preceded by a rapid loss of weight, and the animal shows signs of extreme cyanosis. Rigor mortis sets in rapidly. Post mortem there is an almost complete absence of fat and the skin is infiltrated and vascularized."

Boas postulated two hypotheses viz., that when raw egg white is dried the drying destroys some factor which can be supplied by fresh eggs, dried yeast etc., *or* the drying produces a toxic substance which is neutralizable by some unknown factor in certain foodstuffs. Later it was found that fresh raw egg was just as effective in producing the injury as the dried eggs but that cooking of the egg destroyed the toxic effect.

Boas' egg white injury was studied extensively by Parsons et al (10). During this period of research Salmon and Goodman (11) showed that the syndrome could be produced by fresh raw egg. The Parsons group showed that the syndrome could be produced in the chick, the rabbit and monkeys, as well as in rats, by a diet rich in raw egg white. Schultz (12) produced the syndrome in the dog. It was the Parsons group that demonstrated the presence in raw egg white (fresh or dried) of some factor causing the injury; and the presence in certain foodstuffs of a factor that detoxifies this egg component. From a study of the behavior of this protective factor in the feces of rats on raw and cooked egg white diets Parsons et al in 1940 suggested that raw egg white derives its ability to produce a pathological condition by combining with and holding in unabsorbable form the protective factor.

The study of egg white injury had developed two problems for solution: a) What is the nature of the substance which results in the injury and b) what is the substance that by combining with it renders it ineffective.

To Gyorgy and coworkers (13) goes the credit for the preliminary work in identifying the chemical nature of the protective substance. Because Gyorgy believed that in egg white injury he was dealing with an induced vitamin deficiency, as early as 1931 he called the protective factor vitamin H. "H" was selected, as stated before, because it was the initial of the German word for skin and would therefore suggest a vitamin deficiency which produced skin lesions.

To Eakin, Snell and Williams (14) credit is due for identifying the factor in raw egg white which, by binding biotin, induced biotin deficiency. They called it "avidin" or the "hungry protein". For further details concerning avidin see Section II.

Egg white injury, then, is a manifestation of a biotin deficiency disease produced by the combination of the avidin of the egg with the vitamin biotin, which thus renders the latter non-utilizable by the animal.

Biotin as Bios IIb

Kögl and Tönnis (15) discovered biotin by using a yeast strain known as "Rasse M", and an increased rate of growth under defined conditions as the biological test. Williams, Eakin and Snell (16) used a different strain of yeast and found that sensitivity to biotin increased if the concentration of other nutrilites was so balanced as to make biotin the limiting factor. In general, it appears that most yeasts can synthesize biotin to some extent. Whether or not addition of biotin gives a stimulation depends on the rate of synthesis. For strains of Gebrüder Mayer and "old process" yeast, omission of biotin from the medium seriously impaired growth; for a strain of Fleischmann bakers yeast the omission of biotin was less serious but a marked increased stimulation followed its addition. Leonian and Lilly (17) compared the relative requirements of ten different strains of yeast for 5 nutrilites (biotin, thiamine, inositol, pantothenic acid, and pyridoxine) using 72 hour growth. No strain grew significantly without biotin; all were restricted without pantothenic acid; one was dependent upon thiamine. Interrelations in growth effect were evident; the nutritional need for any one substance was not absolute, and specific requirements depended upon the presence of other nutrilites.

In connection with the relation of biotin to yeast growth, Gyorgy (13) found that the biotin in yeast was present in combined form extractable by autolysis, and the biotin thus freed gave him his first data on its probable molecular size.

In the case of bacteria, biotin is an essential metabolite for some forms, but there are many bacteria which can synthesize biotin readily and, as in the case of yeasts, there are a few forms where synthesis is so slow that stimulation can be produced by added biotin. Knight (18) lists the forms shown in Table 47 for which biotin is an important or essential nutrient. Of these the Rhizobia are of particular interest because Allison and Hoover (19) found that *Rh. trifolii*, *melilotis*, and *leguminsarum* were unable to grow without what they called coenzyme R. This later proved identical with biotin.

In general, then, the role of biotin in the matter of bacterial growth is that of an essential metabolite required for use in some common and fundamental growth processes. Those bacteria that cannot synthesize it or produce insufficient amounts, are dependent on an external source of supply.

In addition to higher animals, bacteria and yeasts, biotin is important for the growth of some of the lower fungi and for at least one insect (Trilobium confusum).

Possible Enzyme Relationship of Biotin

Koser et al (20) noted that the presence of aspartic acid in a culture medium spared the amount of biotin necessary for growth. Glutamic acid had much less effect and 18 other amino acids, including cysteine, had no effect. Pimelic acid was also of no effect. This study was extended (21, 22). The Lyman group (22) showed that with *S. fecalis R.* aspartic acid was not essential if both paba and CO_2 were present; with *L. casei* if CO_2 was present.

In 1944 Summerson et al (23), using liver slices from a biotin-deficient rat and lactate as a substrate, noted a slight rise in both oxygen consumption and RQ on addition of biotin. A similar result appeared with pyruvate but not with glucose as the substrate.

TABLE 47

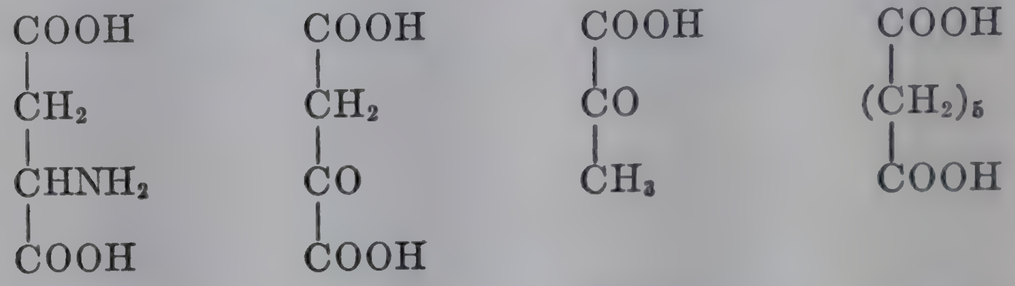
Forms of bacteria for which biotin is an important essential nutrient

(After B. C. J. G. Knight (18) 1945)

Rhizobia
Bact. radicicola IV (Rh. leguminosarum)
Rh. trifolii 205, 209
Rh. meliloti, 131
Rh. leguminosarum
(Other strains synthesize biotin)
Clostridia
Cl. butylicum
Cl. acetobutylicum
Cl. tetani
Cl. sporogenes
Cl. felsineum (carbone)
Other forms
Lactobacillus arabinosus 17-5
Lactobacillus casei
Streptococcus lactis R.
Streptococcus hemolyticus (a group A strain C 2035)
Staphylococcus aureus (some strains)
Brucella abortus
Brucella melitensis
Brucella suis

These results, coupled with the Wood-Werkman observations on the CO_2 fixation reaction (pyruvate plus $CO_2 \rightarrow$ oxalacetate) and the fact that aspartic acid is a transamination product of oxalacetic acid, made it appear possible that biotin might be concerned in the CO_2 fixation route of pyruvate metabolism and perhaps in transamination. (For the chemical structure of these compounds see figure 25.)

This viewpoint was strengthened by Landy et al (24) who showed that either oxalacetate or aspartic acid compensates for lack of biotin in the growth of *L. arabinosus* 17-5; aspartic acid being somewhat more effective than oxalacetate. Also when bicarbonate was used as a source of CO_2, it greatly stimulated growth in the presence of biotin but not in biotin-deficient media. Still further evidence came from Shive and Rogers (25). These investigators studied the effect of biotin inhibitors on the growth of organisms. They showed that aspartic acid or oxalacetate (but not succinic, malic or fumaric acids) decreased the amount of biotin necessary to reverse the toxicity of γ-(3,4 ureylcyclohexl)butyric acid for *L. arabinosus* 17-5. They advanced the idea of a biotin-containing coenzyme acting as a catalyst in the formation of oxalacetate from pyruvate and CO_2. Still later Lichstein and Umbreit (26) showed that *E. coli* cells held at ph 4 and 37° C for 15–60 minutes lose the power to produce CO_2 from aspartic, malic or oxalacetic acids. That ability is restored by the addition of biotin. Their theory is that biotin is localized in an oxalacetate decarboxylase, probably functioning as an enzyme. They also reported that when the deaminases for aspartic acid, serine and threonine, were inactivated by exposure of living cells to M phosphate at ph 4 for 30 minutes at 27–27° C activity was restored by addition of 0.1 mcg. of biotin per ml. of the reaction mix. No other B vitamins produced this effect and this might indicate that biotin has concern in a transamination enzyme system. To date however no such enzyme systems have been identified. More recently Lichstein (52) has given further evidence of a possible coenzyme form of biotin in a yeast extract. This conclusion was based on finding greater biotin activity of the yeast extract compared with assayable biotin and on demonstration that cell-free preparations of aspartic deaminase from ground, vacuum dried preparations of E. coli were stimulated by the yeast extract or by a mixture of biotin and adenylic acid but not by biotin alone.



$$\begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \qquad \begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{array} \qquad \begin{array}{l} COOH \\ | \\ CO \\ | \\ CH_3 \end{array} \qquad \begin{array}{l} COOH \\ | \\ (CH_2)_5 \\ | \\ COOH \end{array}$$

Aspartic Acid — Oxalacetic Acid — Pyruvic Acid — Pimelic Acid

FIG. 25. COMPOUNDS INVOLVED IN THE SYNTHESIS OF ASPARTIC ACID

Avidin, Lysozyme and Biotin

Another possible participation in an enzyme system has been advanced by Meyer (27) and Laurence (28). Lysozyme is a mucolytic enzyme concerned with defense against bacterial invasion. Avidin and lysozyme occur in the same fraction of egg white and in constant proportions; both are basic proteins. Biotin added to preparations containing lysozyme and avidin, according to Meyer, enhanced the lysozyme activity. Laurence suggested that lysozyme is a combination of avidin and biotin; that free avidin is a member of a group of related substances acting as carriers in a system of enzymes in which biotin serves as the prosthetic group.

Further study of lysozyme failed to confirm this viewpoint. Alderton et al (29) found in crystalline lysozyme only 1/1,000,000th of the amount of biotin demanded by the hypothesis that it was the prosthetic group. The avidin content was also small and, in addition, they were unable to confirm Meyer's claim that biotin increased lysozyme activity. This latter difference could have been due to difference in analytical methods.

Biotin and Lipotropic Action

Gavin and McHenry (30) suggested the relation of biotin to fatty livers. They reported that rats on a low protein diet with no vitamin B supplement developed fatty livers on addition of biotin or liver extract in one week unless inositol was given as a supplement. Handler (31) however could find no specific action of biotin but rather an increase in demand for lipotropic factors by stimulation of growth and appetite. (The problem of "biotin fatty livers" is reviewed in Chapter XII, pp. 210.) Present data suggest that the biotin fatty liver hypothesis be discarded.

Relation of Biotin to Lactation and Reproduction

Kennedy and Palmer (32) reported that biotin is one of the factors needed by the rat for successful gestation and birth of viable young and probably a necessary factor for lactation. Black rats are more susceptible to deficiency than albinos. The authors suggested that this would appear to support the view that biotin is essential for rapidly growing tissues (see discussion of relation to cancer). Coryell et al (33) studied the distribution of biotin in human milk. They found the amount insignificant for the first four days after parturition; during the second five days it varied in subjects from 0.24–25.77 mcg. per day and during mature milk production 0.9–10.8 mcg. per day and 2–14 per cent of the intake in milk secreted in 5 day periods. Fifteen to seventy per cent of the intake was excreted in the urine. Variation in the fecal output and synthesis probably explained the wide variation in response.

Biotin in Tumor Metabolism

In line with the suggestion of Kennedy and Palmer (32) cited above, West and Woglom (34) have shown that malignant tumors vary widely from normal tissues in biotin content; that there is a similar difference between embryonic and adult tissues. Experimental skin tumors, for example, contained 400 per cent more biotin than normal skin. On the other hand the biotin content of sarcomas was lower than that of the normal tissues. It has also been reported by du Vigneaud et al (35) that rats subjected to butter yellow-induced tumors could be maintained on a diet that gave them a high degree of protection but that the addition of biotin concentrates broke down this protection.

Such findings raised the question of whether it might be possible to reduce the development of neoplasms by producing a biotin deficiency in the victims. One such way might be by feeding large amounts of egg white and such experiments have been made (36, 37). To date they have been without success. For further discussion of this problem see Section III.

Relation of Biotin to Infections

It is generally accepted that nutritional deficiency from any cause tends to lower resistance to infection. In the matter of specific resistance to a particular type of infection biotin deficiency has been claimed to lower resistance to malaria and to salmonella infection.

The relation to malaria was studied by Trager (38). Trager produced biotin deficiency in chicks and ducks by feeding an egg white diet and followed by infection with large doses of Plasmodium lophurae. He reported that the biotin deficiency decreased resistance to the malarial parasite. Experiments with Plasmodium gallinaceae produced similar results. Kligler et al (39) like Trager produced biotin deficiency by feeding egg white to rats and mice, controls received boiled egg white in which the avidin is destroyed. The biotin-deficient rats showed a markedly greater percentage of organ infection than the controls. Trager suggested that the various species of malarial parasites require different optimal concentrations of biotin in the blood. When the biotin level exceeds a favorable concentration range the multiplication of the parasite may be reduced and the acute infection terminated. Biotin-deficient animals may be less capable of increasing sufficiently their blood biotin levels and as a result be unable to bring infections under control. The possible importance of these findings to control of human malaria is discussed in Section III.

Biotin and Potassium Deficiency

In 1944–45 Smith (40) reported a respiratory paralysis occurring in dogs maintained on a purified diet supplemented with 8 water-soluble vitamins. This affection responded to treatment with biotin. Ruegamer et al (41) induced similar paralytic effects in dogs on the Smith diet, but found them correctible by adequate potassium. Their findings were confirmed by Smith himself (42), but there appears to be an effect traceable to biotin. Biotin exerts a temporary indirect effect but potassium deficiency appears to be the primary factor. How biotin may affect blood level of potassium has not been elucidated.

Biotin and Spectacle Eye

One of the first and most constant of symptoms of biotin deficiency in rats is a denuded area around the eye that gives to it a "spectacled" appearance. This defect had been attributed to another vitamin deficiency (see p. 211) but in 1942 Nielsen and Elvehjem (43) showed that pantothenic acid, corn oil, and inositol were without protective effect while 2 mcg. of biotin per day was both preventive and curative. They also showed biotin deficiency to be responsible for the hind leg paralysis which did not respond to B_2, B_6, or B_2 plus B_6. High fat diet gave a slight protective action but there was no decrease in creatine content and no gross pathology of the nerves.

Biotin and Perosis

Perosis in chicks (also known as hock disease and slipped tendon) has been traced to deficiency of manganese (44), choline (45) and bone phosphatase (46). In 1942 Jukes and Bird (47) and Richardson, Hogan and Miller (48) reported that biotin deficiency could also cause perosis. It is possible that there are still other factors that play a role in the prevention of perosis, but biotin appears to be one that must be added to the list.

Avidin and Ovarian Function

Avidin occupies a unique position in nutrition. By combining with biotin in the intestinal tract it prevents absorption of the vitamin and thus produces a biotin deficiency. Avidin occurs as a secretory product of the mucosa of the oviduct. It has been found in the albuminous portion of all fowl eggs studied and in the egg jelly of frogs. In 1942 Hertz and Sebrell (50) suggested that it might play a role in the embryonic development and in the physiology of the genital tract. Fraps, Hertz and Sebrell (51) investigated this hypothesis and concluded that avidin formation is dependent, either directly or indirectly, on the complete reproduction function of the ovary. Injection of stilbestrol followed by progesterone induced formation of avidin in the magnum. In contrast, progesterone by itself was without effect and only an occasional bird treated with stilbestrol alone showed avidin in the magnum. These observations certainly indicate that avidin is related to ovarian activity but in just what manner remains to be explained.

Summary

The relation of biotin deficiency to human nutrition is reviewed in Section III. The usual manifestations of biotin deficiency in experimental animals are dermatitis and loss of hair, the basis of Gyorgy's "Vitamin H" designation. In rats there is a type of paralysis of the hind limb, in chicks dermatitis and perosis, in monkeys both dermatitis and hair loss. Its possible participation in an enzyme system has been postulated but to date no such system has been identified.

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Section II. Forms and Chemistry of Biotin

The biotin isolated by Kögl and Tönnis (1) from duck egg yolks was called alpha-biotin. The biotin isolated from liver and milk by duVigneaud et al (2) and later synthesized was called beta-biotin. In 1943 Kögl and Ham (3) suggested that since the beta biotin had twice the activity of their alpha biotin for the Rasse M strain of yeast, the two forms probably differed in structure.

This contention has been investigated by Krueger and Peterson (4). They used a sample of Kögl's alpha biotin crystals (25 per cent impure) and Merck's synthetic beta biotin and measured their effect on five organisms (2 lactic acid bacteria, *Cl. acetobutylicus*, a yeast, and a mold). With all of these, taking into account the 25 per cent impurity in the alpha biotin, the alpha biotin gave 90–96 per cent of the activity of the beta form; a difference so slight as to be negligible. They state that there are two possibilities:

1. That the two are chemically identical *or*
2. That they may differ in structure but be similar in activity effect.

Of these two possibilities the first seems most probable.

It is also established that the d-biotin is the active vitamin, the l-form being inactive (5).

For a comprehensive review of how the structure of biotin was developed the reader is referred to the article by Melville (6) in Vitamins and Hormones and by duVigneaud (7) in "The Biological Action of the Vitamins."

Chemical and Physical Properties of Biotin

Biotin occurs in the tissues in two forms; free and combined. Free biotin is water- and alcohol-soluble but relatively insoluble in chloroform, ether, or petroleum ether. It crystallizes from water in long, colorless needles. The ester crystallizes from a methanol-ether mixture in long, thin, platelike crystals.

The possibility of two active forms of free biotin has already been noted, and that viewpoint was supported in the early days of study by finding that the crystals from egg yolk melted at 216° C while those from liver and milk at 230–232° C. The melting points of the esters also differed; alpha at 161.5° C; beta at 166–167° C.

Pure biotin is optically active $(\alpha)_D^{22} = 92°$ for a .3 per cent solution in 0.1N NaOH biotin ester $(\alpha)_D^{22} = 57°$ for a 1.0 per cent solution in chloroform. Biotin has a characteristic absorption in the ultra violet. Various homologs of biotin have been prepared, of these desthiobiotin and dl-oxybiotin are of special interest.

Desthiobiotin

During the work leading to proof of biotin structure a procedure devised for the hydrogenolysis of organic sulfides was applied to biotin methyl ester. The result was a product which was named (8) desthiobiotin. It is formed by the replacement of the sulfur of biotin by two hydrogen atoms. Biologically it showed interesting difference from biotin in activities. It proved to promote growth like biotin for certain strains of yeast, but inhibited growth (antibiotic action) for certain bacteria. Leonian and Lilly (9) have reported that 12 yeasts and four filamentous fungi were able to grow in a desthiobiotin medium while they found it inhibitory to the growth of L. arabinosus, L. casei, Rh. trifolii, and Sordaria finicola. Rubin et al (10) reported desthiobiotin was 50 per cent as active as biotin for S. cerevisae, but with rats made biotin-deficient by feeding egg white or by administering succinylthiazole, dl-desthiobiotin showed only .0001 to .001 the activity of biotin.

Melville et al (8) reported that either biotin or desthiobiotin made available to yeast produced half maximum growth at a concentration of 4.75 × 10^{10}. Also Dittmer et al (11) demonstrated that the ability of yeast to utilize it for growth was because of ability to convert it to biotin.

These investigators grew *S. cerevisae* on biotin-free media to which were added various amounts of desthiobiotin. At the end of the incubation period (16–46 hours) medium and cells were separated and both autoclaved (the medium without acid; the cells with acid). Heating to 120° C with acid destroys 60–80 per cent of the desthiobiotin but does not destroy biotin. Both the autoclaved medium and cells were then tested for growth-promotion effect on yeast and *L. casei*. Results proved that in this treatment the yeast had converted the desthiobiotin into biotin or biotin vitamers. But for amounts of over 0.1 mcg. of desthiobiotin the conversion was not complete: with 1.0 mcg. of desthiobiotin only 20–30 per cent was converted, enough to cause growth promotion of the yeast.

Desthiobiotin, then, appears to promote growth only in organisms that can convert it into biotin; for others it is growth inhibitory.

Oxybiotin or O-hetero Biotin

Hofman et al (12) and Duschinsky et al (13) produced another analogue of biotin. Duschinsky suggested for it the name O-hetero biotin; Hofman, dl-oxybiotin. The latter name appears now preferred. As shown in figure 26, oxybiotin is biotin with the sulfur replaced by oxygen. Pilgrim et al (14) reported it to have one half the activity of d-biotin for *L. arabinosus;* 40 per cent for *L. casei*. Rubin et al (15) found it 25–50 per cent as active for microorganisms as d-biotin but only 5 per cent as active as d-biotin for cure of egg-white injury in rats. They also reported that like biotin it combined with avidin and was inhibited by desthiobiotin. Like desthiobiotin it is convertible to biotin by yeasts.

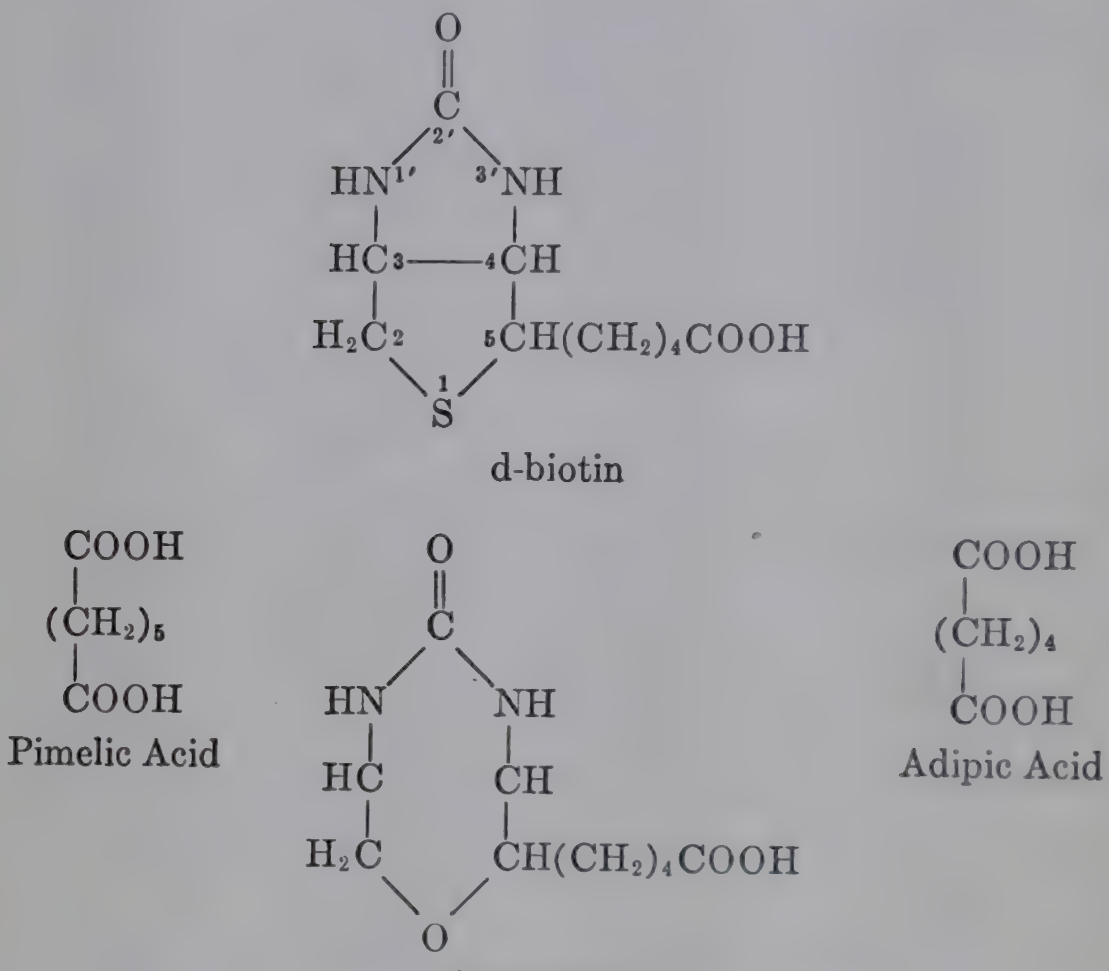
McCoy et al (24) reported study of the effect of dl-oxybiotin on biotin deficient chicks. They found them to show growth response to injections of dl-oxybiotin but without increase in biotin content of the liver, muscle or spleen. Growth response paralelled the amount of oxybiotin injected and the amount found in the tissues. The oxybiotin in the tissues as readily liberated by autoclaving with 5N HCl.

They concluded that the chick utilizes dl-oxybiotin directly; does not convert it to biotin, and that the sulfur atom of biotin is not essential to its biological activity for the chick.

Anti-biotins

In 1944 Dittmer and duVigneaud (15) reported on the properties of biotin sulfone, desthiobiotin, and two imidazolidone carboxylic acids (caproic and valeric). In 1947 (16) they reported on the effect of four imidazolidone aliphatic acids (valeric, caproic, heptanoic, octenoic) on *S. cerevisae* and *L. casei*. In 1945 English et al (17) described the preparation of ureylene benzene and cyclohexane derivatives that possessed anti-biotin activity. (For structure of these compounds see figure 27.)

Some of the findings concerning the behavior of these compounds are shown in Table 48. Incidentally, Dittmer and duVigneaud developed a method of expressing anti-biotin activity as the molar-inhibition ratio, i.e. the number of molecules of the antibiotin necessary to inhibit one mole of biotin. The ratio is determined by measuring the amount of antibiotin necessary to reduce a growth produced by 0.0002 mcg. of biotin to that produced by 0.0001 mcg. of biotin; the smaller the ratio, the greater the activity of the antibiotin.



d-biotin

Pimelic Acid

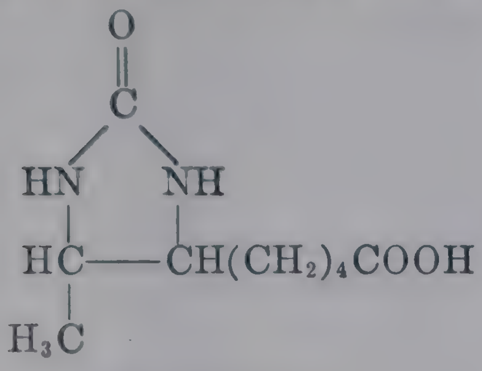
dl-oxybiotin

Adipic Acid

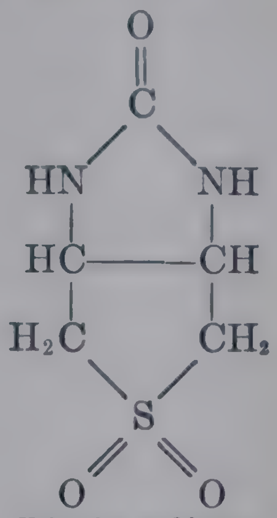
FIG. 26. d-BIOTIN AND POSSIBLE PRECURSORS

Avidin

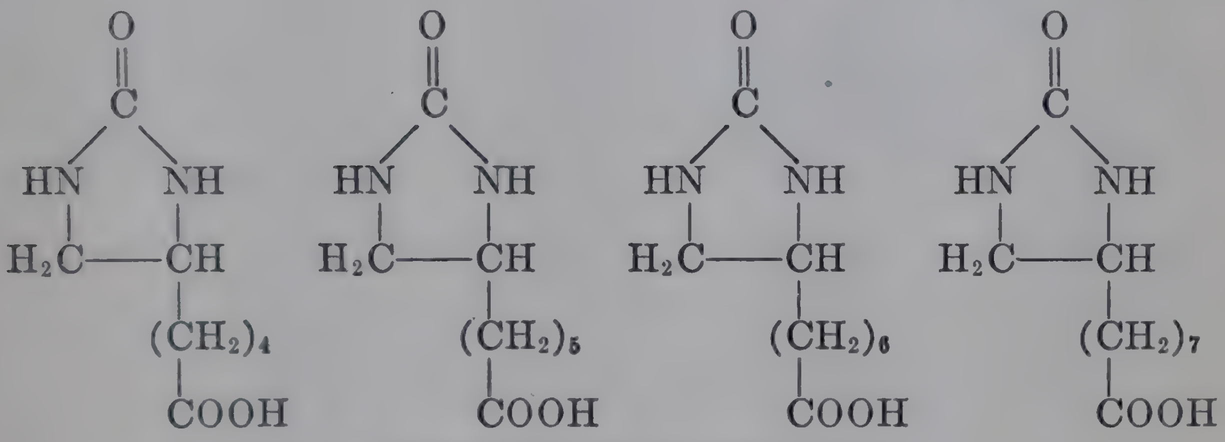
A discussion of biotin chemistry would not be complete without the inclusion of avidin. Avidin (the "hungry" protein) was the first antibiotin substance discovered and is a protein compound in egg white that, by combining with and binding biotin, creates biotin deficiency.



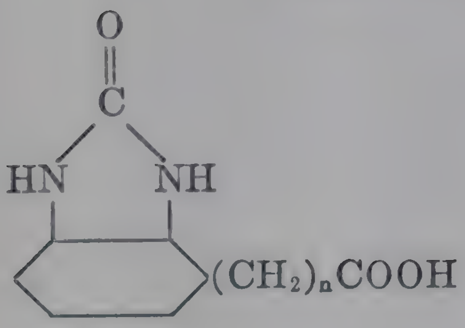
dl-desthiobiotin



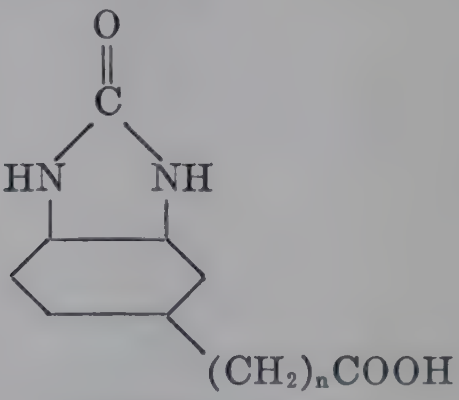
Biotin sulfone



Imidazolidone Carboxylic Acids



Ureylene benzene



Ureylene cyclohexl

FIG. 27. ANTIBIOTINS

In 1940 Eakin, Snell and Williams (17) reported the isolation of avidin from raw egg yolk; the biotin was released by steam sterilization. In 1941 they described a method of separating the less coaguable avidin from other proteins and suggested a molecular weight of approximately 87,000.

In 1944 Pennington, Snell and Eakin (18) reported on the details of the crystallization of avidin. The compound precipitated in fine needles or plates. The amorphous form had an antibiotin activity of 7000 units per gram; the crystalline avidin 4000 units per gram. Elementary analyses reported were as follows:

TABLE 48

Reported activity of various antibiotins

I. After Dittmer and du Vigneaud: Science, **100:** 129 (1944)

Compounds	Growth-promiting activity		Antibiotin activity*		Combines with avidin
	Yeast	L. casei	Yeast	L. casei	
	per cent	*per cent*			
Biotin	100	100	none	none	Yes
Biotin sulfone	0.1	0	none	280	Yes
Desthiobiotin	100	0	none	9100	Yes
Imidazolidone valeric	0.0017	0	none	none	Yes
Imidazolidone caproic	0.0	0	760,000	126,000	Yes

II. After Dittmer and du Vigneaud: J. Biol. Chem., **169:** 63 (1947)

Imidazolidone aliphatic acids	Antibiotin activity* for L. casei
Valeric acid form	52×10^6
Caproic acid form	10^5
Heptanoic acid form	1.6×10^6
Octenoic acid form	10^7

III. After English et al., J. Amer. Chem. Soc., **67:** 297 (1945)

Compounds	n	M.E.C. × 10^4†		Inhibition ratio × 10^{-5}‡	
		Yeast	L. casei	Yeast	L. casei
Ureylene benzene	3	0.625	100	3.1	250
Ureylene cyclohexl I	3	0.003	50	0.015	125
Ureylene cyclohexl II	3	0.003	25	0.015	62.5
Ureylene benzene	4	5.000	25	25.0	62.5
Ureylene cyclohexl I	4	0.006	0.125	0.03	0.31
Ureylene cyclohexl II	4	0.006	0.125	0.03	0.31

* Antibiotin activity expressed as molar inhibition ratio; see p. 000.

† Indicates the smallest amount in moles per liter which produces greater than 50% inhibition of growth of L. casei after 72 hours in a medium containing the minimum concentration of biotin (4.1×10^{-10} M) for normal growth.

‡ Moles of antagonist required to inhibit the growth-promoting effect of one mole of biotin.

Carbon: 43.72–44.26; Hydrogen: 7.60–7.28; Nitrogen: 12.10–12.83; Sulfur: 1.32; Residue: 2.09–0.75.

They considered it a protein with a large carbohydrate moiety.

According to Gyorgy and Rose (19), the whole egg contains an excess of avidin over biotin. The biotin in the yolk is in undialyzable form but biologically active. They reported that biotin is not liberated from avidin by proteolytic enzymes (pepsin, trypsin, pancreatin, papain) nor by incubation with liver, kidney, muscle, or blood. A small part is split by 0.45 per cent H_2O_2. Avidin loses activity as a biotin binder on standing, more readily in dilute than in concentrated solution. Its activity is greater at 38° C than at ice-box temperature. It is destroyed by irradiation with visible light and light destruction is accelerated by the presence of riboflavin. Bringing to pH 1.8 with HCl results in loss of most of its activity. Avidin-bound biotin is not released by acid. Cooking egg white destroys the power of the contained avidin to bind biotin.

As noted in Section I (p. 233) avidin was shown (20) to be present at all levels of the magnum or albumin secretory level of the oviduct of laying hens. It was not present in other parts of the oviduct nor in the atrophic oviduct of the non-laying hen. It is believed to function in the physiology of the ovary in some manner. As an antibiotin avidin inhibits the growth of bacteria that require biotin as a growth factor, but not those which do not require biotin (21).

Biotin Synthesis in the Organism

Tatum (22) postulates that the synthesis of biotin in the organism is through pimelic acid to desthiobiotin and finally to biotin. The insertion of sulfur into desthiobiotin has been demonstrated in yeast (11). It is also considered as established that the attachment of avidin to biotin is through the urea group in biotin. Williams and Fieger (23) have reported that for *L. casei* the inclusion of oleic acid in the culture medium allowed good growth in the absence of biotin and that in the presence of oleic acid no biotin synthesis could be demonstrated. How oleic acid substitutes for biotin is not made clear at this writing.

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Section III. Evaluation of Biotin in Human and Animal Nutrition

Biotin is fairly widely distributed in natural foods (1). Liver, molasses and milk are particularly good sources, but there is a fair amount in meats and vegetables. This fact coupled with the ability of man to utilize the biotin synthesized by his colonic bacteria makes biotin deficiency in man a rare occurrence. It is also the reason why no quantitative daily requirement in diet has been established.

Biotin is excreted in both urine and feces. According to Oppel (2) it is present in the urine in the free form, and in the majority of subjects he studied urinary excretion varied from 20–50 mcg. per day with no sex differences. On an unrestricted diet the elimination varied greatly, but when subjects ate the same diet consecutively the output was remarkably constant. Day excretions were higher than night excretions. Urine did contain some non-avidin-combining biotin compound which stimulated yeast growth, its nature undetermined. That the intestinal synthesis is highly significant was shown by the fact that the total daily excretion invariably exceeded the food biotin intakes, by 3 to 6 times in Oppel's studies.

Gardner et al (3) have reported a study of the elimination of biotin, pantothenic acid, and riboflavin by three healthy young women who subsisted for 10 days on an exclusive diet of mineralized milk supplemented with caramel candy ad lib to supply necessary calories. They took one quart of the milk at each meal. The mineral supplement consisted of 8.5 grams of iron pyrophosphate, 0.4 gms. copper sulfate, 0.4 gms. manganese sulfate dissolved in a pint of water. Table 49 shows the findings. Note that the total biotin excreted was in excess of intake, the pantothenic acid almost the same as the intake and the riboflavin only about half the intake.

In 1948 Oppel (4) reported a further study of biotin behavior in man. He proved that biotin formed in the colon by intestinal bacteria was utilizable. Biotin dissolved in water and introduced per rectum did not cause quite so great an increase in urinary excretion as when given by mouth. It was, however, sufficient to prove absorption from the colon.

TABLE 49

Biotin elimination on a mineralized milk diet

(After Gardner et al. (3) 1943)

Daily intake supplied by the diet	Excretion in		Total	Excess over intake
	Urine	Feces		
115 mcg. biotin	49.5 mcg.	73.4 mcg.	122.9 mcg.	+7.9 mcg.
6.9 mg. pantothenic acid	6.0 mg.	0.41 mg.	6.41 mg.	−0.49 mg.
7.0 mg. riboflavin	3.4 mg.	0.42 mg.	3.82 mg.	−3.16 mg.

Production of Biotin Deficiency in Man

In 1942 Sydenstricker et al (5) reported production of a biotin deficiency in man by a diet containing at least 30 per cent of the total calories in the form of desiccated egg white. It was cured by parenteral administration of a biotin concentrate supplying 130–150 mcg. of biotin per day. The characteristics of the syndrome were a grayish pallor of the skin, atrophy of the lingual papillae of the tongue, mild depression followed by extreme lassitude and sleeplessness, loss of appetite, a slight anemia, and electrocardiograph changes similar to those in thiamine deficiency. One patient developed a maculo-squamous dermatitis of the hands, arms and legs. It was this report that stimulated hope of control of neoplasms by induced biotin deficiency.

Biotin and Neoplasms

In 1942 West and Woglom (6) noted a difference in the biotin content of malignant growths and normal tissues. They found that skin tumors contained 400 per cent more biotin than normal skin. In contrast the biotin content of liver carcinomas (hepatomas) in rat, mouse and man contained less that 50 per cent of the biotin of the normal tissue.

In 1937 Kinositu (7) had shown that "butter yellow" (p-dimethylamino-azo benzene) could be used to produce carcinomas in rats. The effect of biotin on the promotion and reduction of such butter yellow tumors was studied by various workers. It was shown (8, 9) that pure biotin promoted the formation of these tumors in rats and that dietary egg white decreased liver damage and acted anti-carcinogenically in rats fed butter yellow. (8, 9, 10, 11).

These studies and the results of Sydenstricker stimulated hope that by controlling the biotin supply of human subjects a control of neoplasm growth might be possible. Experiments in this direction have been made by Rhoads and Abels (12) and by Kaplan (13). In the former studies two patients were treated (one with a breast cancer and one with chronic lymphocytic leukemia) by giving a diet low in biotin and supplemented with dried egg white. They also used an avidin concentrate. These experiments were continued for a period of 30 weeks. The patients never showed the symptoms described by Sydenstricker and their daily urinary excretion of biotin was variable but within normal limits, indicating adequate intestinal synthesis. The development of the malignancy was not affected.

Kaplan (13) reported a still more intensive study. He used 10 patients:

A and B with myelogenous leukemia; C carcinoma of the stomach with metastases; D melanosarcoma of the leg; E squamous cell carcinoma of the tongue; F fibrosarcoma of the leg; G Hodgkins disease; H cancer of the lung; I metastatic neck cancer; J postoperative carcinoma of the breast with metastases.

The diet given was low in biotin and supplemented with other essential vitamins and minerals. He used the whites of 36–42 eggs daily to produce biotin deficiency and with one patient 2 grams of avidin concentrate. The studies were for periods of six months to a year and in all but three of the patients roentgen ray therapy was used.

Like Rhoads and Abels, in spite of the high egg white intake he failed to get the syndrome described by Sydenstricker et al. He reported that the general condition of the patients improved but this could have been due to control of diet, care and psychic effect. The malignancies were not eliminated. Patient (E) without roentgen ray therapy improved sufficiently to permit talking and there was definite improvement in patient (G), but the disease was not cured. Kaplan expresses belief that the roentgen ray therapy was improved by the use of avidin; in one case of leukemia the irradiation effect was prolonged and was unusually effective in the case of patients (D) and (I).

To date, then, production of biotin deficiency in man or modification of neoplasm content of biotin by use of avidin has not been successful. For detailed discussion of the cancer problem as related to nutrition the reader is referred to the review by Burk and Winzler (14).

Biotin and Malaria

That biotin might be of use to man in controlling the growth of malarial parasites was suggested by the work of Trager (15).

In 1943 Trager reported the following experiments. He used Rhode Island red chickens and Pekin ducks. He first produced in them a biotin deficiency by feeding large portions of egg white and then into these biotin-deficient birds he injected malarial parasites. Ordinarily malarial infection in birds results in a rapid increase of the parasites in the blood, severe anemia, and ultimate death.

Trager found that in birds inoculated with *Plasmodium lophurae* there was a distinctly higher average peak parasite number in biotin-deficient birds than in the controls. Relation of parasite increase to blood biotin level proved interesting. As the infection increased in severity the blood biotin level also increased. Trager interprets this as a mobilization of biotin into the blood stream. According to Trager various species of malarial parasites probably require different optimal concentrations of biotin in the blood. When the biotin level exceeds a favorable concentration range, the multiplication of the parasites may be reduced and the acute infection eliminated. Biotin-deficient animals may be less capable of increasing sufficiently their blood level, and as a result cannot bring the infections under control.

Biotin and Sulfonamides

With the increasing use of sulfa drugs and their effect on the intestinal organisms that synthesize biotin, it could follow that continued use of such drugs might create human biotin deficiency.

Ordinary doses of sulfonamides do not seriously impair the intestinal synthesis of biotin, but the effect varies with the type of sulfa drug and the size and duration of the dosage. Large doses of sulfasuxadine, for example, can almost completely inhibit the synthesis of biotin in the colon. Whether this constitutes an actual menace to man is not certain but severe biotin deficiency in animals by use of sulfa drugs has been produced in less than six weeks.

Biotin Deficiency in the Monkey

When seeking a deficiency syndrome production that may closely resemble that expected in man, we look to the monkey as the favorite experimental animal. In 1937 Lease and Parsons (17) produced a syndrome in the monkey (Macacus rhesus) by feeding a diet which for the first few days contained 20 per cent of egg white and then was stepped up to 30 per cent. Two of the monkeys developed roughening and thinning of hair and a bald spot after two months on the diet. At this time the egg white content was increased to 40 per cent. The eyelids became inflamed and the monkeys got listless and stayed huddled up. A steamed egg white diet or a dose of aqueous liver extract cured the condition.

Between 1943 and 1945 Waisman et al (18) also produced and studied biotin deficiency in the monkey. In their first experiments they used a basal diet of sucrose, purified casein, salt mixture, corn oil, and cod liver oil with a daily supplement of 9 vitamins (B_1, B_2, niacin, B_6, Ca pantothenate, choline, paba, inositol, and ascorbic acid). This was fed to 11 rhesus monkeys. Some of these got a daily supply of 10 mcg. of biotin from the start, others after development of nutritional failure. Addition of biotin did not prevent this nutritional failure which they attributed to folic acid deficiency. Nutritional failure was manifested as loss of appetite, loss of weight, and leucopenia. But while the biotin supplement did not correct the nutritional failure symptoms, they got convincing evidence that it was necessary for the maintenance of a normal coat. Monkeys on the basal diet plus folic acid lost their coats in 2 to 7 months and 20 mcg. of biotin per day brought about complete restoration in several of the monkeys in four months.

In the second series of studies (Macaca mulata monkeys), three methods of inducing biotin deficiency were used. For chronic biotin deficiency the same basal biet described above was used with various liver preparations as sources of folic acid. The biotin content of these liver sources was assayed microbiologically. Hair loss was found to follow ingestion of less than 10 mcg. of biotin daily in these sources. When the liver source of folic acid supplied more than 12 mcg. of biotin daily no fur changes occurred in a 28 month period of observation.

The biotin deficiency induced by egg white produced symptoms identical with those described by Lease and Parsons but the feeding period before their appearance varied from 21 to 105 days with different monkeys. The dermatitis was more severe than that produced in the chronic deficiency experiment.

The same acute deficiency was also produced by adding to the basal diet three per cent sulfasuxadine and a 1 per cent liver extract. (Without the sulfasuxadine this diet did not produce nutritional failure.) The effect of the sulfasuxadine could be counteracted by giving 20 mcg. of biotin daily.

Biotin and Domestic Animals

As in the case of other B vitamins, ruminants (cattle and sheep) in general synthesize an adequate amount of biotin and hence are independent of dietary supply (19). Cow's milk is a good source of biotin and its content throughout lactation and in various seasons has been reported by Lawrence et al (20). They found an extreme variability in the biotin level during the colostrum period. There appeared to be an initial rise in biotin concentration from an average of 13 mcg. per liter to about 27 mcg. per liter in the first three days of lactation, followed by a decrease to a normal level of about 18 mcg. per liter. It did not appear to be affected by seasonal variations. There is in milk a correlation coefficient between biotin and pantothenic acid levels of 0.29 which is apparently not due to random distribution. Pasteurization does not destroy the biotin in milk.

In the case of chicks it has already been noted that biotin deficiency may be one of the causes of perosis. Deficiency also produces a dermatitis similar in appearance to that observed in pantothenic acid deficiency.

The following is quoted from the National Research Council Bulletin (21):

> "The lesions first appear in about three weeks, though the time may vary. The bottoms of the feet become rough and calloused and may be severely affected before mandibular lesions appear. As the syndrome progresses the entire bottom of the foot becomes encrusted and hemorrhagic cracks appear. The toes may become necrotic and slough off but the top of the foot and leg usually show only a dry scaliness. The mandibular lesions which first appear in the corners of the mouth spread to include the area around the beak, and the eyelids eventually become swollen and stick together. In contradistinction to these symptoms, the lesions in pantothenic acid deficiency are first evident in the corners of the mouth and eyes and only in extreme cases do the lesions of the feet become severe.
>
> Biotin has been reported to be a factor necessary for the prevention of perosis in chicks and turkeys. Turkey poults exhibit symptoms very similar to those described for chicks when fed a biotin deficient ration.
>
> Feeding mature fowl a biotin deficient ration causes reduced hatchability while egg production in not adversely affected, indicating that the requirement for the production of hatching eggs is much higher than that for maintenance of good health and egg production. In hens, no dermatitis similar to that of chicks fed a biotin deficient ration has been observed."

The Committee on Animal Nutrition (21) recommends 45 mcg. of biotin per pound of feed for starting checks; 70 mcg. per pound of feed for laying and breeding hens.

For swine, Hogan and Anderson (22) note that in their synthetic rations they have always included biotin (30 mcg. /100 gms. feed) but it is uncertain whether it contributed improvement to the diet.

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CHAPTER XV. CHOLINE

SECTION I. FUNCTIONS OF CHOLINE

Choline is not a new chemical compound. It was recognized as a component of the sinapin of mustard seed by von Babo and Hirschbrunn (1) in 1852, and as a component of lecithin by Strecker (2) in 1862. Its significance as a possible vitamin and nutritional essential followed from a study of the behavior of depancreatized dogs used in the development of insulin. While insulin prevented diabetes in the depancreatized dogs they later developed fatty livers and in 1932 Best et al (3) showed that the fatty livers could be prevented by the inclusion in the diet of crude egg yolk lecithin or beef pancreas. The factor in the lecithin responsible for the transport of fat from the liver was shown to be its choline component. There followed a study of the role of choline. As a result of this study choline was shown to have a dual nutritional significance: first, because of its intact molecule it was used in the formation of phospholipids and acetylcholine and second, by its use as a supplier of labile methyl groups for transmethylation reactions.

Details of the discoveries that established the values of choline have been covered in several reviews (4, 5, 6, 7) to which the reader is referred. The findings are summarized in the following pages.

Choline as a Lipotropic Factor

It has long been held that one method by which the fat of the liver gets into circulation is by conversion into phospholipids and into choline containing lecithin in particular. In 1942 Griffith (5) summarized the viewpoint as follows:

> "The relation of choline to fatty livers appears direct in view of the recognized role of the choline phospholipids, especially lecithin, in the transport of fatty acids. However, the exact mechanism of fatty acid transport and utilization are still unknown so that a conservative view of the role of choline in lipid metabolism would be that of Best (8) viz., 'that dietary choline increases slightly the phospholipid content of the liver and this promotes the transport of fatty acids, as phospholipids, from the liver to other tissues or promotes the utilization of fatty acids in the liver itself.'"

That choline's role in lipotropic action involves the production of phospholipids by actual combination of choline with other components of the phospholipid appears now established by a number of contributing studies.

In 1936 Welch (9) reported the feeding of arsenocholine and its effect on the arsenic content of the resulting phospholipids. The arsenocholine had a lipotropic effect similar to choline itself and from the amounts of arsenic found in the phospholipids Welch drew the conclusion that the arsenocholine actually entered into combination with the other components of the phospholipid molecules. In 1942 Welch and Landau (10) reported farther on the behavior of arsenocholine. The fact that it was lipotropic and antihemorrhagic for kidneys but *inactive as a methyl donor* convinced them that the intact choline molecule is utilized in the synthesis of lecithin and probably other phospholipids containing choline.

Further evidence that choline acts by stimulating the formation of phospholipids was obtained by Chaikoff and his collaborators (11) with the use of radioactive phosphorus. Their earlier results gave evidence that choline accelerated the formation of phospholipids in the liver and other tissues, the site of most active formation being the liver. Their finding was confirmed by Stettin (12). He labelled the choline molecule with heavy nitrogen, isolated the choline from the bodies of his labelled choline fed animals and found a high concentration of his heavy nitrogen in the choline phospholipids.

In later studies of the Chaikoff group, radioactive phosphorus was used in following phospholipid metabolism. Dogs were injected intravenously with radioactive sodium diphosphate in isotonic solution. Some of the dogs received large doses of choline chloride a short time before the phosphate. Others received no choline and served as controls. The specific activity of the choline containing phospholipids of the liver increased far more greatly in six hours in the choline treated dogs than in the controls. By excluding the liver from circulation these workers also showed a marked slowing of the rate of phospholipid turnover.

From these combined data it appears certain that phospholipids are formed from choline in the liver; that the liver is concerned with both the synthesis and transport of plasma phospholipids; and that choline acts not only as a component but also as a stimulator of liver phospholipid formation.

The amount of fat in the diet is also a factor in the behavior of choline. Fishman and Artom (13) showed that while the level of phospholipids in the livers of rats on a high fat diet was increased by feeding choline, on low fat levels the addition of choline had little effect.

Hemorrhagic Kidney Degeneration

In 1939 Griffith and Wade (14) noted hemorrhagic degeneration of the kidneys in young male rats fed an a-lipotropic diet. Christensen (15) and Gyorgy and Goldblatt (16) have reported the following characteristics of this condition: vascular congestion and degeneration of the tubules were the principal lesions; congestion of the peripheral cortical capillaries and the capsular blood vessels producing enlargement and a deep red appearance; hemorrhage found only in the capsule and at the edge of the cortex. Also in the more severe lesions the glomerular and other blood vessels were congested; the tubules in the deep part of the cortex and in the outer part of the medulla were necrotic and always filled with casts. Such kidney changes could be prevented by additions of 1–2 mg. of choline daily to the basal diet.

Griffith in 1941 (17) reviewed the possible explanation of these kidney effects. They are believed to follow from the effect of choline on phospholipid turnover. Best (6) puts the present viewpoint as follows:

> "It is reasonable to suppose that the kidney effect, like the liver change, is exerted through the mechanism of an altered rate of phospholipid turnover. This may not involve transport of phospholipid from the kidney but may be concerned with the nutrition of the kidney cells."

Welch and Landau (10) made a similar suggestion, viz., that fatty livers develop when there is deficiency of choline for the synthesis of compounds involved in fat transport, and renal hemorrhage occurs at a critical growth period when there is an acute deficiency of compounds required for the synthesis of material essential to cell structure.

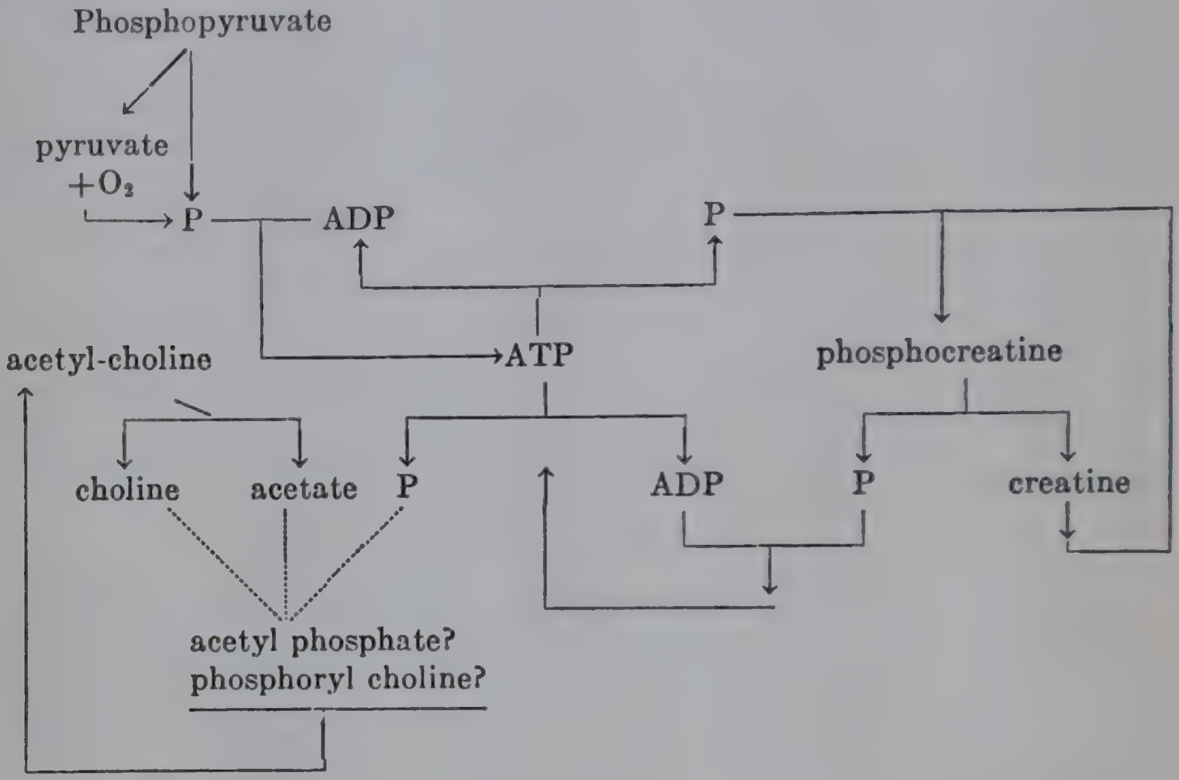
Acetyl-choline Formation

Another important function of choline involving the use of the intact choline molecule is in the formation of acetyl-choline, the substance that makes possible the transmission of impulses from efferent nerves to effector cells. According to Nachmansohn (18) the choline for this synthesis is derived from the phospholipids of nerve tissue and its acetic acid from pyruvic acid. At least two enzymes are concerned in its formation and hydrolysis, choline acetylase (19) and a specific cholinesterase (20). It is also possible that thiamine and carboxylase are also involved to produce the necessary acetic acid from pyruvic acid (21). Figure 28 shows the schema of acetyl-choline formation according to Nachmansohn (18).

Other Lipotropic Factors; Methionine

In 1935 Best and Huntsman (22) noted that the protein casein produced a lipotropic effect. This finding was confirmed by Channon et al (23) and led to a study of the effect of various amino acids. In 1937 Tucker and Eckstein (24) explained the lipotropic effect of casein as due to its methionine content. They also confirmed the report (25) of the effect of cystine as an anti-lipotropic factor in rats on a low casein, high fat diet.

Confirmation of the lipotropic effect of methionine soon followed (26, 27), and study to explain its mechanism was inaugurated. The most important advance came from duVigneaud et al (28), who suggested that methionine exerts its lipotropic action by contributing its methyl group for the formation (synthesis) of choline, a transmethylation reaction later proven (29) to occur. It is now established that methionine yields a methyl group which by combining with ethanolamine produces choline. (See figure 28.) In 1932 Best and Huntsman (30) showed that glycine betaine also had lipotropic activity. This was confirmed (31, 32, 33, 34) and also that it had only about one third the activity of choline due to the fact that only one methyl group was available for transmethylation. Its action, like methionine, is in the conversion of ethanolamine to choline. The lipotropic activity of glycine betaine led to study of other betaines, but apparently only alanine betaine (31) and cystine betaine (33) exhibit lipotropic activities.



N.B. A.T.P. = adenosine triphosphate
A.D.P. = adenosine diphosphate
P = energy rich phosphate bond

FIG. 28. FORMATION OF ACETYL-CHOLINE AFTER NACHMANSOHN (18) 1945

Choline as a Methyl Group Source

An important corollary to this formation of choline by transfer of CH_3 groups from methionine appeared in the finding that the reverse action was possible and that choline, by supplying methyl groups, could bring about the production of methionine. In 1932 Butz and duVigneaud (34a) reported that methionine loses its methyl group when boiled with strong sulfuric acid. From the reaction mixture a new sulfur containing amino acid (homocystine) was isolated. It was in 1939 that duVigneaud et al (35) first reported that while rats were unable to grow on a synthetic diet containing homocystine or homocysteine in place of methionine, growth resulted when choline was added. The explanation offered (36) was that choline donated its methyl groups to homocysteine and formed methionine. This discovery, now amply confirmed, gave to choline a function quite distinct from supplying the intact molecule, namely ability to produce, by donation of its methyl groups, amino acids essential to growth and nutrition. The following diagram illustrates the relations of the compounds shown to be involved in such transmethylations:

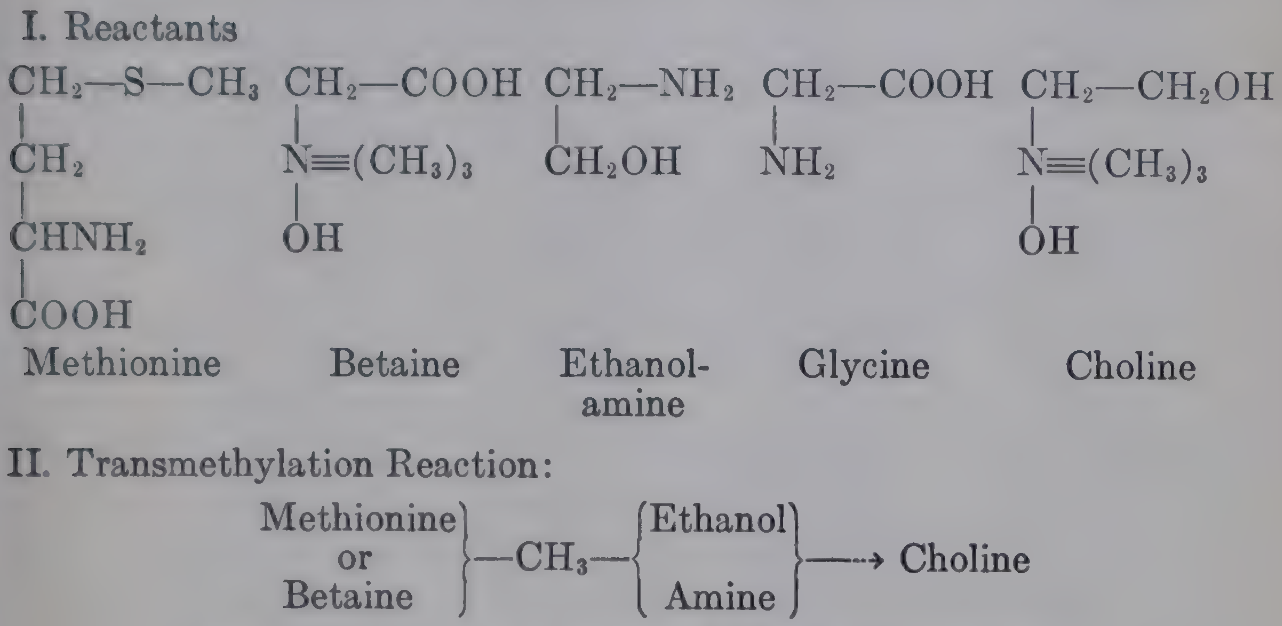
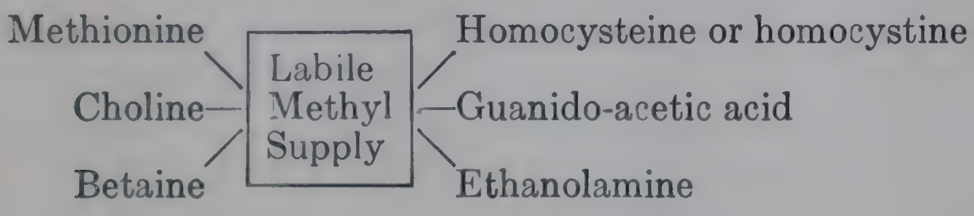


FIG. 29. FORMATION OF CHOLINE BY TRANSMETHYLATION



The Effect of Cystine

The reports (24, 25) that cystine, unlike methionine, produced a large increase in liver fat in rats on a low casein, high fat diet have already been mentioned. At first it was suggested that there was a true antagonism between cystine and choline. Griffith's explanation, (37) now generally accepted, is that cystine brings about an increased utilization of food, raises the metabolic level nearer to normal and thus creates an increased demand for choline or other lipotropic factor; that its action is *non-specific*.

The Lipotropic Action of the Pancreas

It will be recalled that in the early study of liver fat in depancreatized dogs, removal of the fat was shown possible by feeding either crude lecithin or beef pancreas (3). In 1936 Dragstedt et al (37) reported finding in pancreas a substance which they called "lipocaic" and that this substance relieved and prevented accumulation of liver fat in depancreatized dogs. Also it required much more choline than was present in an effective amount of pancreas to produce an equivalent effect. Entenman and Chaikoff (39) have granted that there is present in pancreas some other factor than choline involved in the lipotropic action of the pancreas.

The most recent explanation of the effect of pancreas, aside from any choline that may be present, is that it supplies a proteolytic enzyme that liberates methionine from the protein of the diet and choline is in turn perhaps synthesized by transmethylation from the methyl groups of the methionine. The enzyme thus is accountable for producing choline, methionine or both to which the lipotropic effect is due.

Choline as a Growth Factor

McKittrick (40) has reported a new procedure for measuring growth effects and has used this method (41) to study the growth producing interrelations of choline, methionine and betaine in chicks during the fourth week of life. Without going into detail as to the method used, he reported the limit below which the choline content of the diet could not be reduced, regardless of the level of methionine, without reducing growth below optimum level and similarly the limit below which the methionine content could not be reduced regardless of the level of choline. These limits, which he calls the "essential levels," were approximately 0.1 per cent of the diet for choline and 0.5 per cent of the diet for methionine. These essential amounts were not replaceable one by the other. But, for optimum growth, the essential levels of choline and methionine had to be supplemented by a replaceable amount of choline or methionine or an equvalent mixture, e.g. 0.25 per cent choline plus 0.45 per cent methionine.

Excess of methionine depressed the rate of chick growth from a high of 6.18 per cent per day on 0.94 per cent choline plus 0.5 per cent methionine to a low of 3.35 per cent per day on 0.94 per cent choline plus 1.28 per cent methionine in the diet. But this growth retarding effect of excess methionine was counteracted by providing a methyl acceptor in the form of glycocyamine (guanido-acetic acid) to take up the excess methionine methyl groups; serine produced the same effects. Methylation of glycocyamine by methionine methyl groups presumably formed homocysteine which does not suppress growth unless choline is added. In the absence of homocysteine and at the essential level of methionine, choline chloride at levels up to 1.6 per cent did not suppress growth.

McKittrick's studies are unique in not only supporting the general belief that choline's principal role in promoting growth is its action as a methyl donor but also are the first studies to show a quantitative relation between choline and methionine in growth promotion. McKittrick's studies dealt only with chick growth studies, but one would expect similar relations between choline and methionine in growth effects on other animals.

As a component of acetyl-choline and phospholipids, as a methyl donor in the production of essential amino acids such as methionine, choline would obviously have a direct relation to the normal metabolism and growth progress of animal life. It has also been shown essential for lactation in the rat (42) and the hamster (43).

Choline and Prevention of Liver Damage

Dutra and McKibbin (44) have described the pathological changes in puppies on a choline-deficient diet. They found the livers pale yellow or pinkish yellow. They contained much lipid material in vacuoles but irregularly distributed. The vacuoles, too, varied in size from small to large, the latter being more numerous. Fatty degeneration and infiltration of the liver with atrophic changes of the thymus were the most prominent signs. Either choline or dl-methionine prevented these changes; 100 mg. of choline or 0.7 mg. of dl-methionine per 100 grams of ration.

Microscopic examination of brain, heart, lung, spleen, pancreas, adrenals, kidneys, testis, ovary, stomach, intestine, eye and costa chondral junctions revealed no abnormalities.

The authors, however, are of the opinion that more than simple fat infiltration is the result of choline or other lipotropic factor deficiency. To settle this point they subjected the animals to standard liver function tests.

Puppies that had been on choline-deficient diets for 68 days were selected and 200 mg. per cent of choline chloride administered. They were restored to normal by this dosage within ten days. The contrast in liver function responses were as follows: In the ten day period the plasma phosphatase dropped from 1348 to 357 Bodansky units; brom-sulfathalein retention was reduced from 28 to 7 per cent; total plasma cholesterol rose from 52 to 154 per cent and plasma cholesterol from 34 to 101 per cent; prothrombin time returned to normal. Of all these indices of liver function the phosphatase level was the slowest to return to normal. The choline supplementation was also accompanied by striking increases in food consumption and body weight. They also devised tests to try and show differences in transmethylation activity of the livers but these tests were rather unsatisfactory.

Choline, then does not simply prevent infiltration of fat but actually helps maintain normal liver functions.

Choline and Liver Cirrhosis

In 1942 Gyorgy and Goldblatt (45) and Daft et al (46) suggested that a combination of cystine and choline might be of value in the prevention of cirrhosis. Studies of such use will be discussed more in detail in Section III, but it is mentioned here as another possible function of choline. In the reports referred to, cystine alone was found to aggravate the condition, but in combination with choline was preventive. The dosage of cystine plus choline (1.5 gms. cystine; 3.0 gms. choline per day) has been reported beneficial when combined with a high protein, low fat diet (47).

Choline and Bone Formation

The production of perosis in chicks as a dietary deficiency effect has already been noted (see p. 233). But according to Jukes (48), choline as well as manganese is necessary to prevent perosis in chicks and turkey poults. Though the gross symptoms of perosis caused by choline deficiency are not distinguishable from those produced by manganese deficiency, Hegsted et al (49) showed that in the choline deficiency type the bone phosphatase was normal. Jukes claimed approximately 0.2 per cent choline required for normal bone growth and perosis prevention.

Choline and the Production of Hemolytic Anemia

Does choline have a pharmacological effect quite apart from its action as a nutritional factor? Davis (50) raised that question by reporting that with simultaneous feeding of high fat and choline chloride to dogs he got a sudden large destruction of erythrocytes. Hypochromic anemia resulted from long term feeding of progressively increasing doses of choline chloride with elevated fat intakes. The surprising thing about these results was that Davis got the hemolytic effects with amounts of choline much below that used to prevent fatty livers.

In 1947 Davis (51) suggested that the effect was due to a vaso-dilator action of choline and had earlier prevented the effect by atropine. The vasodilator action was believed to exert an inhibiting action on erythropoiesis in the bone marrow. Clarkson and Best (52) were unable to get the result in dogs noted by Davis. In 1946 Davis (53) stated that the hemolytic effect could be produced by acetyl-choline as well as by choline and that concurrently with the production of anemias the cholinesterase activity of the dog serum fell to a low value. With the institution of anti-pernicious anemia therapy the red cell count, hemoglobin, and esterase activity of the serum returned to normal levels, folic acid having the same effect as liver extract. In interpreting these findings, Davis has suggested that increase in concentration of acetyl-choline as a result of decrease in cholinesterase produces a dilatation of the vascular network of bone marrow with increase in marrow oxygen tension. This increase in oxygen tension in turn inhibits maturation of cells of the erythroid series.

These reports raised the question of possible harm in choline therapy especially, as Davis' harmful effects came with amounts below those used in normal procedures. For example it had been shown that, following pancreatectomy, 35 mg. per kilo of body weight per day could be used for dogs, while Davis got his harmful effect with 8 mg. per kilo per day. Since folic acid and perhaps other factors in the diet are necessary to prevent this hemolytic effect it may be that danger from choline dosage occurs only when the diet lacks adequate amounts of the antianemia factors. Moosnick et al (54) have reported that they were unable to produce anemia in human subjects by choline dosage.

Toxicity of Choline

There are no quantitative data on human usages, but from studies of animal responses it has been assumed that human requirements for choline lie between 0.1 and 0.2 per cent of the diet, or 1.5–3.0 grams of choline per day for adults. Again, though the distribution of choline in natural foods has not been very extensively measured, a rough estimate (55) indicates that the daily diet of man could be estimated to supply from 1.5 to 4.0 grams of choline.

Again, on the basis of animal studies, the LD_{50} dose of choline for man appears to be in the order of 200–400 grams and minimum toxic effects are expected at between 15–70 grams of choline per day. Incidentally, 6 grams per day has been used in attempted treatment of cirrhosis of the liver in man without toxic effects.

Choline and Pigmentation

In 1941 Lillie et al (56) described a yellow, fluorescent, acid-fast "ceroid" pigment that was present in great amounts in the liver of rats in which cirrhosis was produced by dietary means. Dam and Mason (57) reported that a diet containing 20 per cent cod liver oil brought about acid-fast pigment in the adipose tissue of young rats and that the pigmentary changes were prevented by the administration of alpha-tocopherol. These results suggested that the diets used to produce cirrhosis were also deficient in vitamin E, and when Endicott et al (58) included alpha-tocopherol in their experimental diet they were able to produce cirrhosis without ceroid pigment.

But Earle and Victor (59), in studying the problem, found that when choline was added to a diet of 15 rats receiving a 5 per cent l-cystine, low protein diet, only 3 of the 15 rats showed ceroid pigment in their livers. From this they drew the conclusion that choline had partially inhibited ceroid formation. The relation of choline to ceroid pigment still remains obscure.

A type of pigmentation that may be the result of the pharmacological action of choline has been reported by Higgins et al (60). They noted in a large percentage of young animals a bronzing of rat hair, induced by choline chloride. The reddish brown pigment, adherent to the hair, first appeared around the base of the tail. Within a few days after administration of choline chloride was begun it had gradually extended forward to involve the whole animal. Supplementation of the diet with yeast brought the color back to normal in about two weeks, and animals whose diet included yeast from the start did not develop the pigmentation, even when given large amounts of choline chloride daily. In general a protein diet supplemented with either yeast or liver appeared to protect the animals against this pigmentation effect. Apparently here is another case of a choline chloride effect dependent on the absence from the diet of some essential protective factor.

Relation of Choline to Other Members of the Vitamin B Complex

Results such as the Davis hemolytic anemia effect and the Higgins bronzed pigmentation effect and their prevention by certain members of the B complex in the diet has stimulated study of the relation between choline and these other B-complex members. McHenry (61) has reported a relationship between thiamine and choline in the production of fatty livers. In thiamine-deficient animals, when no thiamine is provided, fat does not accumulate in the liver in the absence of dietary lipotropic factors unless liberal amounts of fat are fed. With thiamine and even without fat in the diet fat is formed and deposited in the liver. This fat is however rich in glyceride, low in cholesterol, and small amounts of choline prevent its deposition. Gavin and McHenry (62) also found that riboflavin and pyridoxine also increase liver fat deposition, but not unless thiamine is supplied. Engel (63) states that addition of calcium pantothenate to a synthetic diet containing thiamine, riboflavin, pyridoxine and choline caused a 100 per cent increase in liver fat. These effects of thiamine are explained on the basis that the fat is formed from carbohydrate, a conversion in which the thiamine plays an essential role (64). The action of pyridoxine is explained as being concerned in the conversion of protein into fat (65). Both pyridoxine and thiamine appear essential for the formation of glycogen from protein.

These possible relationships have been reviewed in detail by McHenry and Cornett (66) and by Griffith (67) and the reader is advised to consult these expositions for information in this field.

Relation of Choline Need to the Type of Fat in the Diet

It has already been made evident that a number of other components can affect the production of fatty livers by feeding a diet low in choline or methionine. Channon et al (68), for example, reported that the inclusion of saturated fatty acids in a given type of diet led to more deposition of fat than the inclusion of solid unsaturated acids. In general it is known that the greater the amount of fat in the diet the greater chance of fatty livers, though it is also true that fatty livers can result from diets low in fat or containing no fat at all (62).

In order to get more data on the effect of saturated fatty acids Stetten and Salcedo (69) have studied the effect of the length of the carbon chain on fatty liver formation. In their study they fed the acids as ethyl esters at 35 per cent of the diet. Each was fed for a 2 week period. Some proved definitely toxic: 15 out of 16 rats receiving ethyl laurate died. Ethyl stearate killed 2 rats and ethyl caprylate 4 out of 9 recipients. The effect of the esters in rats that survived the test also varied greatly. The authors give the following data:

In the stearate group (3 rats) mean liver fatty acid content was 3.2 per cent.
In the palmitate group (4 rats) mean liver fatty acid content was 9.4 per cent.
In the myristate group (4 rats) mean liver fatty acid content was 17.2 per cent.
In the caprylate group (5 rats) mean liver fatty acid content was 4.4 per cent.
In all other groups mean liver fatty acid content was 7.0–8.0 per cent.

The behavior of myristate is outstanding but it must be borne in mind that these fatty acids were fed as ethyl esters, not as glycerides. It may be wrong to conclude that the glycerides in which form these fatty acids normally exist in diets would show similar effects. The results do show, however, that not only the quantity but the type of fat in the diet can influence the requirement of choline or other lipotropic factor.

Conclusion

There have been other reported effects of choline deficiency. It has been said a deficiency lessens the toxicity of ethylene dichloride (70). Neoplasms have been reported to occur in the liver, lungs, and other tissues as a result of prolonged choline deficiency (71). Some of these effects will come properly for discussion in our Section III. It is evident, however, from what has preceded that failure to produce certain products essential to nutrition by either lack of sufficient intact choline molecules or lack of methyl groups for transmethylation reactions will explain the basic relation of choline to observed results of deficiency.

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Section II. Forms and Chemistry of Choline

Structurally choline may be described as a substituted ammonium hydroxide (tri-methyl-hydroxy ethyl-ammonium hydroxide). In combination with fatty acids it forms lecithins and when acetylated produces acetylcholine. The structural formulae for these compounds is shown in figure 30.

Properties of Choline

Pure choline is obtainable in crystalline form but the product is usually sirupy because of its hydroscopic nature. It readily decomposes and when heated breaks down to trimethyl amine and glycine with small amounts of beta-diethyl amino-ethanol and dimethyl vinyl-amine. The pure product has a caustic bitter taste.

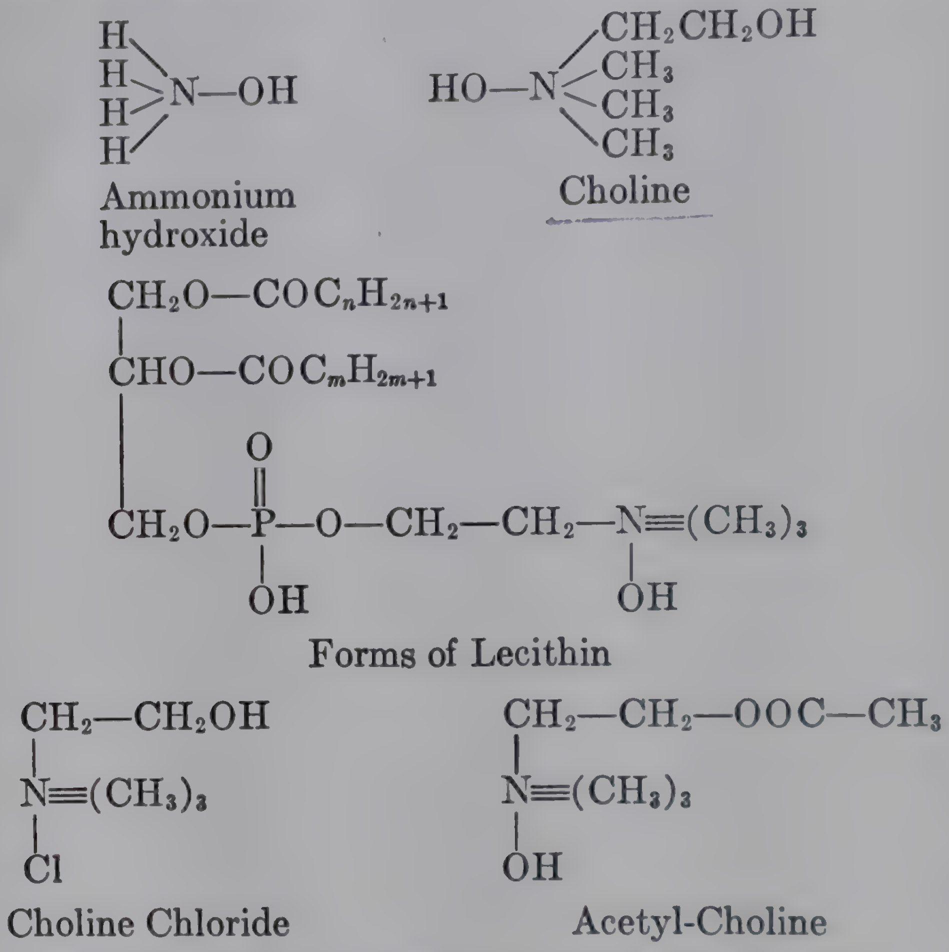

FIG. 30. STRUCTURE OF CHOLINE AND RELATED FORMS

Empirical formula: $C_5H_{15}NO_2$; molecular wt. 121.13

Choline is soluble in water, in methyl and ethyl alcohol, and in formaldehyde; only slightly soluble in dry amyl alcohol, dry acetone and chloroform; insoluble in dry ether, petroleum ether, benzene, toluene, carbon disulfide, and carbon tetra chloride. It is a strong base. Choline chloride is the form usually employed for therapy.

Properties of Choline Chloride

Empirical formula: $C_5H_{14}ONCl$; molecular wt. 139.57

Contains 86.78 per cent of choline. It is in the form of colorless, deliquescent crystals; very soluble in water and alcohol. The water solution is practically neutral. Usual dose is 0.6 grams in 240 cc. of sterilized physiological salt solution.

Properties of Acetyl-choline Chloride

Empirical formula: $(CH_3)_3NCl—CH_2—CH_2—O—OCCH_3$; molecular wt. 181.59

Contains 79.9 per cent acetyl choline; 66.7 per cent choline. It is a white, odorless, hygroscopic, crystalline powder; very soluble in *cold* water and alcohol. Usual dose is 40–200 mg. subcutaneously or intramuscularly.

Choline substitutes: In certain species (1, 2, 3) and probably in man, methionine can replace choline in the diet. Apparently this is caused by biosynthesis of choline through union of methyl groups from the methionine with amino-ethanol which in turn may be formed from glycine. Betaine (4) can substitute for methionine as a source of labile methyl groups. Proof of these reactions has been accomplished by the use of isotopes and the following examples may be cited:

a) Glycine rich in N^{15} has been isolated from the tissues of rats fed with N^{15} labelled betaine providing definite evidence of the transfer of nitrogen to betaine in the rat (4, 5).

b) When glycine labelled with N^{15} was fed to rats the amino-ethanol isolated from the body phosphatides had an isotope concentration 49 per cent as high as that of glycine isolated from body proteins, evidence of the conversion of glycine to amino-ethanol (5).

c) Studies with rats fed N^{15} tagged amino-ethanol (5) showed 41 per cent of the choline in the phosphatides was formed by the methylation of amino-ethanol, presumably by methyl groups supplied by methionine (6).

d) Rats fed methyl-diamino-ethanol labelled with deuterium in the methyl group produced choline containing a significant amount of deuterium in its methyl groups (7).

e) Formation of lecithin by choline was demonstrated by noting the entrance of N^{15} tagged choline into the tissue lipids (8).

Arsenocholine can substitute for choline in the prevention of fatty livers and hemorrhagic kidneys but not as a source of methyl groups (10, 11).

Tri-ethyl choline is also lipotropic and antihemorrhagic but is not a source of labile metyl groups (12, 13)

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Section III. Evaluation of Choline in Human and Animal Nutrition

That choline plays an important role in animal nutrition has been fully established. A deficiency leads to fatty livers in most species of animals, to hemorrhagic degeneration of kidneys in young rats, to perosis in chicks and turkeys, and to cirrhosis of the liver in rats, rabbits, dogs and perhaps man.

Its place in human nutrition is still unestablished but it has been used clinically in the treatment of human liver cirrhosis and for some other abnormal conditions.

The estimated requirement for man based on animal comparisons has already been stated (p. 257) to be 1.5–3.0 grams per day with indications from distribution in food studies that failure to supply this amount in the average American diet would be rare (1). Measurement of the excretion of choline by man does not give satisfactory data on which to base requirements. Only very small amounts (less than 1 per cent) are excreted in the urine following dosage according to Johnson et al (2) and fecal eliminations are still lower. It is also known that choline availability is intimately linked with the protein intake.

Choline and the Treatment of Human Liver Cirrhosis

Observations on dogs, rats, and rabbits (3, 4, 5, 6) have shown that cirrhosis may develop with prolonged feeding of a diet low in choline. These observations led naturally to attempts to cure human cirrhosis by choline therapy. The results of experiments have been variable. As Jukes (7) puts it:

> "This may be partly explained by the fact that cirrhosis progresses to a shrunken and fibrotic condition of the liver in which there is little possibility of reversal toward normal."

Some of the trials that have been made are as follows:

1. Broun and Muether (8) reported treatment of patients with hepatic cirrhosis for more than two years with one gram of choline chloride daily and a diet high in protein, low in fat and cholesterol, and supplemented

with vitamins A and D. In most of the patients ascites disappeared and the liver decreased in size.

2. Fleming and Snell (9) treated fifty patients with portal cirrhosis by giving them a high protein, high carbohydrate, low fat diet supplemented with vitamins A, D, and the B complex. Within a year 30 of the patients had died. However of the 20 survivors 11 were in good health, their ascites had disappeared and they appeared to be making good clinical recovery. They suggest, in line with Jukes' statement, that improvement is possible only when hepatic damage is not too far advanced.

3. Russakoff and Blumberg (10) noted definite improvement in 7 out of 9 patients given supplements of choline in addition to a high protein, high carbohydrate, low fat, high vitamin diet as evidenced by clinical and laboratory findings. Enlarged livers were considerably reduced in size, ascites diminished or disappeared. serum proteins increased, the hemogram improved, prothrombin time decreased, and liver function tests became normal. In their treatment choline chloride was used in dose of from 1.5 to 6 grams daily.

4. Goldstein and Rosahn (11) used choline alone and choline plus inositol on four patients; diet high in protein, high carbohydrate, low fat. Improvement was noted in all four cases.

5. In 1946 Beams (12) reported a study undertaken to evaluate the importance of a combination of choline and cystine in the treatment of liver cirrhosis. He summarized his results as follows:

> "a) Twenty patients with cirrhosis of the liver who had ascites were treated with a high protein, low fat diet supplemented with yeast and a combination of choline and cystine.
>
> b) Twelve of the 20 patients with livers that were not enlarged showed no response to the therapy, whereas 7 of the 8 patients with large livers made a good recovery from liver decompensation.
>
> c) A comparison of the treated group of patients with large livers with a similar group of fifteen patients who were treated with only a high protein, low fat diet supplemented with yeast, indicated that the choline and cystine had a favorable influence in the treatment of cirrhosis.
>
> d) The striking difference in the response of patients with large livers compared with patients with livers not enlarged suggest that the combination of choline and cystine is effective where fatty changes in the livers is suspected."

Such results give definite encouragement to the use of choline as a possible means of preventing and curing human liver cirrhosis, at least if given early enough and with an adequate diet.

Choline and Infectious Hepatitis

Use of choline and of methionine in the treatment of infectious hepatitis has been tried but with conflicting results (13, 14, 15, 16). There is need for much more research on this problem.

Choline Deficiency and Neoplasms

Copeland and Salmon (17) have described the occurrence of liver neoplasms of the nature of adenocarcinomas in 15 out of 50 rats that survived on a choline deficient diet for 8 months or longer. Also of growths in other tissues, e.g. hemangio-endotheliomas up to 5 cm. in diameter, were observed in the peritoneal and subcutaneous fat in ten per cent of the rats. The lungs of 30 per cent of the rats showed nodules, the larger masses similar to primary medullary carcinoma. Of the 50 rats with cirrhotic livers 3 developed retroperitoneal growths of the sarcoma or fibrosarcoma type. No such lesions were seen in litter mate controls receiving 20 milligrams of choline daily.

Choline in Domestic Animals

The only domestic animals that appear in danger of choline deficiency are chicks, hens and turkeys. These birds develop perosis when the choline content of the diet is deficient. This was pointed out by Jukes (18) in 1940 and confirmed by Hegsted et al (19) and Hogan et al (20) in 1941. In 1942 Record and Bethke (21) reported further evidence indicating that choline is an essential growth factor for poultry as well as perosis preventive, and that approximately 0.15 per cent of choline had to be added to the ration in the form of the chloride to give maximum results. They also noted that while methionine added to the ration increased growth it did not prevent perosis.

The Committee on Animal Nutrition (22) makes the following recommendations regarding manganese and choline:

> For starting chicks: 25 mg. manganese and 0.7 gms. choline per lb. of feed.
> For turkey poults: 25 mg. manganese and 0.9 grams of choline per lb. of feed.
> For laying and breeding hens and turkeys 15 mg. of manganese but no definite allowance of choline.

However it is agreed that for mature birds choline deficiency increases mortality, lowers egg production, and increases abortion of egg yolks from the ovary.

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CHAPTER XVI. FOLIC ACIDS

Section I. Functions of the Folic Acids

In 1941 Mitchell, Snell and Williams (1) reported that using *S. Lactis R.* as a test organism they obtained from spinach an acid nutrilite in nearly pure form. They stated:

> "This acid, or one with similar chemical and physiological properties occurs in a number of animal tissues of which kidney and liver are the best sources. It is widespread in the biological kingdom. Mushrooms and yeast are good sources. It is especially abundant in green leaves of many ferns, including grass.
>
> Because of this fact, and since we have obtained what appears to be a nearly pure chemical entity, we suggest the name 'folic acid' (Latin: folium, a leaf). . . . Folic acid stimulates the growth of *L. casei* under the same conditions as the factor reported by Snell and Peterson (2) in 1940 and recently reported to be isolated by Stokstad (3)."

The product obtained by Snell and Peterson (2) was isolated from a norit adsorption fraction of liver extract. It was this liver extract factor that was further purified by Stokstad (4) and because it stimulated the growth of *L. casei*, he called it 'L. casei Factor'.

The primary similarity of the spinach concentrate and the liver L. casei factor was that both stimulated the growth of two organisms; *L. Lactis R.* or *S. fecalis R.* and *L. casei*. This similarity in behavior suggested that the two substances were chemically identical, but to date the spinach concentrate has never been purified completely.

The discovery of folic acid and liver L. casei factor also clarified some other discoveries. In 1938 Stokstad and Manning (5) presented evidence that chicks required a dietary factor for growth which they tentatively named 'factor U'. In 1939 Hogan and Parrott (6) also reported the existence of an unidentified factor which they called B_o to indicate that it was a member of the vitamin B complex and also necessary for chicks. Comparison of these factors with L. casei factor suggested that they were possibly identical and also that L. casei factor was what Day had designated (7) as the monkey vitamin 'M'.

Actual clarity came with the successful synthesis and chemical identification of liver L. casei factor by the Lederle laboratory group (8) in 1945. Variation in numbers of glutamic acid molecules attached to the pterin-paba base explained the existence of different forms of folic acid. Folic acid today means Pteroyl glutamic acid (9); Fermentation L. casei factor is Pteroyl triglutamic acid (10); B_c Conjugate is Pteroyl hepta glutamic acid (11). Still another form originally designated as S.L.R. factor is now called rhizopterin (11, 12), or formyl pteroic acid. The structure of these forms is shown in figure 31 and discussion of their properties in Section II.

Characteristics of Folic Acid Deficiency

While detection and differentiation of forms of folic acid are based on the growth response of micro-organisms (*S. fecalis R.* and *L. casei*) it is its function as a hematopoietic agent that has commanded special attention.

As a hematopoietic agent folic acid is preventive and curative of forms of macrocytic anemia. In hyperchromic, macrocytic anemia the red cells are larger and contain more hemoglobin than normal; the hemoglobin being increased and the number of red cells decreased. In folic acid deficiency the site of erythropoiesis, the bone marrow, is affected. The character of the bone marrow changes have been summarized (13) based on a report by Endicott et al (14) as follows:

> "The symptoms were leucopenia or a decrease in the normal number of leucocytes in the peripheral blood; granulocytopenia or lack of granulocytes, the cells intermediate in maturity between the progranulocytes and the meta granulocytes. The condition results from failure of normal bone marrow process. The bone marrow in this case shows defective development (hypoplasia) with general cellular depletion, especially of the myeloid series. The erythroid series is also usually depleted even though distinct anemia is not found. Administration of folic acid produces a striking, immediate response both in the bone marrow and in the peripheral blood; in the bood by an increase in hematocrit, total leucocyte, polymorphonuclear, eosinophile, lymphocyte and normoblast counts; in the bone marrow myeloid proliferation and erythroid hyperplasia. The bone marrow later returns to normal after a temporary over-compensation."

Hyperchromic macrocytic anemia has been found in quite a wide range of conditions. Pernicious anemia, sprue, nutritional macrocytic anemia, anemia following partial gastrectomy, are the conditions on which the use of folic acid has mainly been tested. In fact the first evidence of its value came from its use in pernicious anemia (15) and sprue (16). However, as will be explained later, while folic acid does have hematopoietic effect in cases of pernicious anemia, it is not identical with the liver extract anti-pernicious anemia factor.

Other Postulated Hematopoietic Factors[1]

Prior to the identification of folic acids as Pteroylglutamates there had been a number of factors postulated that later proved either identical with a folic acid or acted with it.

Factor 'U'

The factor that Stokstad and Manning (5) called factor U they reported essential to chick growth. When they attempted to rear chicks on a semisynthetic diet depending for its water-soluble vitamins on crystalline thiamine and a whey adsorbate and filtrate, growth was retarded. Addition of yeast increased the growth rate. Wheat middlings, bran and alfalfa leaf meal were almost as good as yeast and rice bran was a fair source of the essential factor. Corn and molasses were poor sources. They believed at the time that they had discovered a hitherto unknown vitamin but today it seems probable that the activity of these natural products was due mainly to what later became B_c conjugate or Pteroyl hepta glutamic acid.

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[1] It is of interest to note that as early as 1931 Funk and Lewja (Bruxelles Medical, 12, 1931) reported fractions from yeast and liver with definite effect on rat anemia.

Factor B_c

Special interest attaches to factor B_c, first postulated by Hogan and Parrott (6) in 1939, in that it ultimately proved to be identical with the liver L. casei factor, namely Pteroylglutamic acid. It was in course of their studies on synthetic diets for chicks that Hogan and Parrott first devised a diet that produced anemia. They then sought for a factor which, when added to the ration, would cure and prevent the anemia and reached the conclusion that the factor was a hitherto unknown vitamin of the B-complex group. Chicks lacking this factor developed a macrocytic anemia and were subnormal in weight.

In 1942 Mills et al (17) noted that a concentrate of Snell and Peterson's (2) norit eluate factor from liver not only promoted the growth of chicks on a purified diet (18), but also prevented the development of anemia and suggested that vitamin B_c might be identical with the norit eluate factor. About a year later B_c as a crystalline compound was actually isolated from liver (19). It produced approximately half maximum growth of *L. casei* at a concentration of 0.00005 mcg. per ml. of culture medium, and had a similar potency for *S. fecalis* (20, 21). It was both antianemic and growth promoting for the chick.

In 1944 Campbell et al (22) reported that a deficiency of B_c in the diet also interfered with feathering. One hundred mcg. of B_c in the ration was stated to be necessary for normal growth and normal leucocyte count; 40 mcg. in the ration to maintain a normal thrombocyte and erythrocyte count. Comparison of crystalline B_c with synthetic L. casei factor demonstrated their chemical identity.

Vitamins B_{10} and B_{11}

It has already been noted that Hutchings et al (18) and Mills et al (17) found in a concentrate of the norit eluate factor something essential for the chick. If this factor or factors were not provided the chicks grew slowly, were anemic and failed to develop normal feathering. Growth was also better on the solubilized liver from which the eluate was made than on the eluate itself, which suggested that there were unidentified vitamins in the liver not identical with B_c. In the search for these factors Briggs et al (23) prepared a number of fractions from the norit eluate and by fractional precipitation with alcohol obtained two concentrates with different action. One fraction appeared essential for feathering and was called vitamin B_{10}; the other was essential for growth and labelled B_{11}. Neither could be identified with B_c by assay with *S. fecalis*. Work has been done toward further concentration of these factors but complete separation and identification has not been attained at this writing. Pfiffner and Hogan (25) commenting on these studies say:

> "It is entirely possible that practically all of the activity observed in the various fractions are due to the presence of vitamin B_c. However, since the rate of growth by chicks as reported by Campbell et al (22) is inferior to the rate observed when crude vitamin carriers are included in the ration, one would suppose that there is at least one additional unrecognized vitamin."

Factors S and R

In 1932 Kline et al (26) showed that the antidermatosis vitamin is destroyed by long continued exposure to dry heat, and Bauerfeind and Norris (27) attempted to determine whether the mature fowl as well as the chick required this vitamin. As expected they found that chicks that consumed the heated diet grew slowly and developed the dermatosis. Supplementing this diet with a rice bran filtrate prevented the dermatosis and improved growth but weights were still subnormal. It appeared that the heating had not only destroyed the dermatosis-preventing vitamin but also some unrecognized factor. This factor appeared to be present in liver and yeast, to be soluble in water, adsorbable on fullers earth, and not destroyed by heating in an autoclave. Schumaker and Heuser (28) reported that the alcohol precipitate of a water extract of yeast was a good source of the factor. When used to supplement the heated diet, this alcohol precipitate or rice bran filtrate produced a slight increase in the hatchability of the hen eggs but a combination of the two produced marked increase in egg hatchability.

In 1940 Schumaker (29) reported attempts to concentrate the alcohol precipitate factor, and reached the conclusion that it contained at least two unrecognized vitamins. An acid extract of yeast was precipitated with 10 volumes of alcohol and filtered. The factor that remained in the filtrate they called 'R'; that carried down in the precipitate 'S'. Addition of both to the heated diet resulted in normal growth. Only slight increase of growth was noted when S or R were added separately.

Because the basal ration was deficient in choline, Hill, Norris and Heuser (31) improved their original diet by addition of choline. Record and Bethke (30) showed that the addition of either choline or factor R increased the rate of chick growth. When both were added there was another increment in growth; but they got no growth response to factor S. Hill et al (31) confirmed their finding that growth was not accelerated by factor S alone but did get maximum growth with factor R alone, perhaps because it was contaminated with some S.

Since there was no correlation as determined by *S. fecalis* assay, between the folic acid content of any of the yeast fractions and their growth promoting activity, they believed that neither factor S or R was identical with folic acid.

On a basal diet of natural foodstuffs the chicks were not anemic, but when a synthetic type of ration was used to impose more rigorous conditions, chicks developed anemia and few survived the experimental period. Addition of factor R to this diet produced normal hemoglobin and slightly subnormal weights. With factor S weights were still lower and chicks anemic. Combination of S and R resulted in chicks normal in all respects.

It is now known that assay with *S. fecalis* does not detect the presence of B_c conjugate (Pteroyl hepta glutamic acid) unless there is a preliminary treatment with a conjugase; hence the conclusion that these factors are not some form of folic acid is not absolutely conclusive and factor R may contain B_c conjugate.

Vitamin M

In 1935 Wills and Stewart (32) reported producing of a macrocytic anemia in monkeys as the result of feeding a diet common among the poor people of Bombay. As the anemia developed the monkeys lost weight, became inactive and lost hair from the tails. The red cells decreased to about one third normal, the hemoglobin to one half normal and the color index was reduced to about 20 per cent. White cells also decreased to one third normal. The disease was cured by 'marmite' (Autolysed yeast) and also by a liver extract *but not by the liver fraction used to cure pernicious anemia.*

In 1938 Wills and Evans (33) reported a similar anemia in men and women which, like the monkey anemia, responded to crude liver or yeast extracts but not to the antipernicious anemia liver fraction.

In this country Day et al (34) fed to monkeys a diet similar to that used to produce cataract in rats. The monkeys did not develop cataract but died of a nutritional deficiency disease characterized by anemia. leucopenia, gingivitis and diarrhea. Since this condition was corrected by dried brewer's yeast, it was concluded that the preventive factor must be a member of the vitamin B complex group. A similar effect was produced by feeding the monkeys Goldberger's pellagra producing diet and was not corrected by niacin (35). Pending identification the investigators called the unknown factor vitamin 'M'. Day (36) has reviewed these studies and summarized the results as follows:

> "Young rhesus monkeys (and apparently other macaques) when given a diet presumably adequate with regard to calories, minerals, and protein develop a syndrome characterized by anemia, leucopenia, oral lesions, diarrhea, and a lowered resistance to infection, especially infection of the intestinal tract. The syndrome can be prevented by yeast or crude liver extract but the feeding of all the pure vitamins has proved ineffective. There is some evidence that xanthopterin may be partially effective in preventing the deficiency disease. Some data are available on the distribution and properties of the factor, which has been termed 'Vitamin M'. It is not known whether this factor is required by primates other than the genus Macaca."

When the B_c crystals of Pfiffner et al (9) and the fermentation L. casei factor became available, Day and coworkers (37) reported the probable identity of vitamin M and folic acid. However, more recent studies have shown that, when monkeys are fed highly purified rations deficient in riboflavin and folic acid, a type of anemia develops which responds only when fresh liver or raw milk is given (38). The distribution and partial fractionation of this new monkey anemia-corrective factor has been reported by Ruegamer et al (39). The method of inducing this syndrome consisted in omitting a vitamin such as riboflavin from the diet until the monkey lost weight. At that point riboflavin therapy was instituted with the result that the monkey would respond suboptimally to the supplement. If a supplement of certain foods fed at this point brought about a weight and hematological response, it was concluded that they must contain the new factor. Since high levels of folic acid, antipernicious anemia factor, l-lysine, and dl-tryptophane failed to produce the same result the new factor could not be folic acid and must be an unknown vitamin M.

Fresh liver and raw milk proved richest in the new factor; whey solids, yellow corn, raw potato and carrot were poor sources. Approximately 4 grams of fresh liver per day were required by the monkey, and fresh liver was selected for fractionation studies. In their report (39) Ruegamer et al state that ethanol, methanol and acetone extracts of fresh liver were all active and a tenfold concentration was obtained by the norit elution method using a norit adsorption of the alcohol extract. While folic acid (PGA) does correct certain features of monkey anemia, no form of folic acid yet isolated fully accounts for its full protection. Further information awaits progress in the isolation of this newly postulated vitamin M, sometimes called the Wills' factor.

Alpha and Beta Pyracin

It will be noted from the preceding that, with increasing availability of pure forms of folic acid, evidence also accumulated to indicate that they are not the only factors concerned in hematological effects. In 1944 while trying to improve the microbiological assay of folic acid Scott et al (40) reported that when pyridoxine was treated with hydrogen peroxide a preparation was obtained that promoted the growth of *L. Casei*. The compound appeared to have antianemia effect for chicks and accelerated the growth rate of the first week. The compound was identified as the lactone of 2-methyl-3-hydroxy-4-hydroxy-methyl-5-carboxypyridine. This name was shortened to alpha-pyracin and its isomeric 4-carboxypyridine to beta-pyracin.

According to these investigators a combination of fermentation L. casei factor (Pteroyl triglutamic acid) with either of the pyracins completely prevented anemia in the chick and accelerated the growth rate, the beta-pyracin being more effective on growth than the alpha form.

However, in 1946 Scott and coworkers (41) stated that if synthetic folic acid (Pteroyl glutamic acid) was given in the amount of 100 mcg. per 100 grams of ration, it produced a hemoglobin level that was not enhanced by pyracin.

Others were unable to confirm the earlier results. Briggs et al (42) could get no response from chicks in the matter of growth, hemoglobin formation, or feathering with alpha pyracin. Campbell et al (43) failed to get any complementary action from beta-pyracin when fed in combination with folic acid (PGA) to growing chicks.

Folic Acids are not Identical with Pernicious Anemia Agent

Evidence that the folic acids are different from liver extract antipernicious anemia agent is indicated by their failure to prevent or cure the neurological symptoms which usually accompany the blood changes in pernicious anemia patients, and also from the failure of liver extracts rich in the antipernicious anemia agent to show appreciable content of folic acid when assayed by microorganisms.

In Spies' review (15) of the clinical value of folic acids are these two statements:

> "In November 1945 Vilter et al initiated a study to test the efficacy of folic acid in maintaining persons with Addisonian pernicious anemia. Each of the twenty four patients selected for study had been maintained under their observation for a number of years on liver extract. Nine months later the study was still in progress but the investigators do not believe it is possible to state with certainty whether or not the oral administration of folic acid can replace the parenteral liver extract in the maintenance of such patients. Synthetic L. casei factor (PGA) certainly does not always prevent the development of neural disturbances that occur frequently in persons with pernicious anemia. Studies such as these must be conducted for a number of years in order to provide us with the important information needed on this point."

And again in the same review:

> "It would seem well to consider that synthetic folic acid (PGA) functions as part of an enzyme system. It is well to keep in mind that the active principle in liver extract

is not synthetic L. casei factor or any of the substances mentioned in this review (Pteroyl triglutamic acid, thymine, xanthopterin etc.). When this substance is obtained in pure form, it will probably be more powerful than any of the substances now available."

Elvehjem (44) puts the situation as follows:

"The activity of pteroylglutamic acid was first demonstrated during the latter part of 1945 and the extensive clinical studies have been summarized by Spies and coworkers (15, 45), Darby (46) and Cartwright (47). Darby has also described the effect of Pteroylglutamic acid on the gastro-intestinal manifestations of sprue and related syndromes. Since commercial preparations of the classic pernicious anemia factor contain no folic acid (48) and since a pure substance, vitamin B_{12}, which is active in very small amounts in the treatment of pernicious anemia has now been isolated (49) it is clear that the effect of folic acid may not be direct or that its effect is closely related to additional factors."

Vitamin B_{12}

In 1948 Rickes et al (49) have reported that:

"A crystalline compound which in microgram quantities has produced positive hematological response in initial tests in patients with Addisonian pernicious anemia has been isolated from the liver." (See chapter XIX) and references (50) (51) (52) (53) (54) (55) (56) (57) (58).

Action of Folic Acids on Various Organisms

The discussion of the place of folic acids in human nutrition is reserved for Section III. The following data show its requirement by certain lower organisms:

The Rat: Like the human being, the intestinal bacteria of the rat synthesize a utilizable folic acid. Hence, to produce folic acid deficiency in the rat it is necessary to supplement a folic acid-free diet with sulfa drugs or a folic acid antagonist.

The earliest study reported (59) used sulfaguanidine to suppress intestinal synthesis and Teply et al (60) have reported that dextrin is the nutrient ingredient in the ration producing maximum intestinal synthesis in the rat of both folic acid and niacin. Also excess of niacin increases folic acid synthesis and excess of folic acid increases niacin synthesis. These syntheses were prevented by addition of 2 per cent of Phthalyl sulfathiazole to the diet. Lactose and milk powder also had stimulatory action on the intestinal synthesis of folic acid.

Another expedient to produce rat folic acid deficiency has been to use a folic acid antagonist (61).

The public Health Group (62) has given much information on the effect of folic acid deficiency in the rat. In addition to granulocytopenia, leucemia, and anemia, folic acid deficiency has been shown to retard growth and impair reproduction and lactation (63, 64), reduce utilization of biotin and pantothenic acid (65) and to produce spleen infarcts (66). Rodney et al (67) have also reported that folic acid deficiency lessens the ability of the livers of such rats to oxidize tyrosine and Higgins et al (68) found evidence of myeloid stimulation by folic acid following gastrectomy.

Petering and Delor (69) failed to find thymine a substitute for folic acid in the rat when fed in amounts equivalent to 2.1, 3.5, 4.2, and 7.2 mcg. of folic acid and it was without effect on growth or any part of the blood picture.

According to Kornberg et al (70) Pteroyl triglutamic acid is just as effective for the rat as Pteroylglutamic acid on a molar basis and Asenzo (66) puts the minimum daily requirement at 5 microgram of PGA.

The Mouse: In 1944 Nielsen and Black (71) reported folic acid and biotin essential nutrients for the mouse. The biotin deficiency resulted in alopecia. As in the case of the rat deficiency became more acute with addition of 0.6 per cent of sulfaguanidine to the ration.

The mouse has also provided another interesting differentiation between the behavior of two folic acids; according to the Leuchtenbergs et al (72) Pteroyl triglutamic acid produced resistance to breast tumors in mice but this effect was not evinced by Pteroylglutamic acid.

The Guinea Pig: Woolley and Sprince (73) reported that several factors were necessary for the guinea pig which they labelled GP 1, 2, 3. Their G.P.1 has been proved identical with folic acid (PGA).

Woodruff and Darby (74) found that guinea pigs on a scorbutogenic diet containing 5 per cent added l-tyrosine had an unusually high urinary excretion of tyrosine derivatives and keto acids. Administration of 15 mg. of folic acid (PGA) or 10 mg. of ascorbic acid for four consecutive days markedly decreased these urinary levels, another bit of evidence of the relationship of folic acid to the oxidation of tyrosine.

The Dog: In 1933 Rhoads et al (75) noted that dogs on a black tongue-producing diet developed an ulcerative stomatitis, leucopenia and granulocytopenia. In 1945 Krehl and Elvehjem (76) reported on the response of dogs to niacin when maintained on a low niacin diet. This ration contained 0.64 mcg. of niacin per gram of diet. Eighteen dogs were placed on this diet and 10 received in addition a folic acid concentrate prepared from solubilized liver extract and administered in 1.5 ml. amount per dog per day.

After niacin deficiency had developed, 5 of the animals maintained on the basal diet alone were given 25 mg. of niacin; to this dose the weight response was 400 to 700 grams. In contrast, giving this amount of niacin to dogs on the basal plus the folic acid concentrate produced 1000 to 1850 grams weight response.

Following the initial response to the niacin dosage, the dogs that had not received the folic acid lost weight rapidly and failed to respond to a second niacin dose. The dogs that had received the folic acid responded repeatedly with weight gains on niacin administration and did not exhibit the severe mouth lesions characteristic of black tongue. Also, there was a trend toward higher hemoglobin white cell counts in the blood of the folic acid dogs.

Krehl et al (77) repeated the experiment but used 25 mcg. of synthetic PGA. Again they got repeated responses in weight to niacin and when the folic acid was removed from the diet the same dosage of niacin produced only one additional growth response and death ensued. They interpret these findings, coupled with the fact that anemia was less severe in the folic acid dogs, as indicating a requirement of folic acid by dogs. The studies also suggested that black tongue may be a complex deficiency disease in which the symptoms of niacin deficiency predominate in some animals, and those of folic acid deficiency in others. This may also apply to pellagra in man. Seeler and Silber (78) however could find no evidence of folic acid requirement by the dog.

Fox and Mink: Schaefer et al (79) and Elvehjem (80) have reported that folic acid is required by both fox and mink, but that they also require some factor or factors, present in raw milk and fresh liver in addition to all the known crystalline vitamins. In the absence of these factors which were separable from liver in a methanol soluble and methanol insoluble fraction anorexia, loss of body weight, depigmented and matted under fur, suboptimal hemoglobin and reversal of the neutrophil leucocyte ratio appeared. Livers were light colored and fatty. These results suggested the need of the Ruegamer factor (43).

For the foxes, yeast containing 30–32 mcg. of Pteroyl heptaglutamic acid was not as effective as a folic acid source, but became effective in a water yeast extract after action of hog kidney conjugase.

Insects: To the animal organisms requiring folic acid must be added at least one insect. Goldberg et al (81) have reported folic acid essential to the mosquito for pupation.

Lactic acid Bacteria: In addition to *S. fecalis* and *L. casei*, folic acid is required by *L. helviticus*, *L. delbruckii*, *Propioni bacterium pentosaceum*, and the tetanus bacillus.

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SECTION II. FORMS AND CHEMISTRY OF FOLIC ACID COMPOUNDS

In Table 50 are listed the important forms of folic acid, its derivatives and their relative activities. The structure of these compounds is shown in figure 31.

Of these compounds three are of special interest since they are the forms that occur in foods. These three are Pteroyl glutamic acid, usually designated as folic acid or PGA; Pteroyl triglutamic acid or Pteroyl glutamyl glutamyl glutamic acid; Pteroyl hepta glutamic acid or Pteroyl hexaglutamyl glutamic acid. As has been previously stated, the original folic acid concentrate from spinach (1) has never been completely purified but is believed to be mainly PGA.

PGA, Pteroyl glutamic acid, (Folic acid, Vitamin B_c, Liver L. casei factor)

In 1943 Stokstad (2) reported purification of Snell and Peterson's (3) norit eluate factor and gave the product the name of Liver L. casei factor. Like the original spinach folic acid (1), it stimulated the growth of both *S. fecalis R.*[1] and *L. casei*. In the same year Pfiffner et al (4) announced the isolation and purification of vitamin B_c from liver, and later (5) this compound was found chemically identical with the Pteroyl glutamic acid synthesized by Angier et al (6) in 1945.

In 1947 Pfiffner et al (7) reported in detail on the properties of their purified B_c and Stokstad et al (8) has reported similar data on the Liver L. casei factor isolated from liver. In 1948 there were also reported (9, 10. 11) other methods of synthesis of Pteroyl glutamic acid and discussion (12) of its structure. That structure is shown in figure 31. Its chemical descriptive name is:

N-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]
-1(+)glutamic acid

[1] In the earlier literature *S. fecalis R* is called *S. Lactis R*. Niven and coworkers have demonstrated that *Streptococcus Lactis R.* is an enterococcus, specifically *Streptococcus fecalis.)*

As shown in the figure it consists of three parts; a pterin group attached through para-amino-benzoic acid to glutamic acid.

TABLE 50

Relative activities of folic acid compounds

(After C. W. Waller et al., J. Amer. Chem. Soc., **70**: 19, 1948)

Compounds	Number of glutamic acid	Amounts to produce ½ max. growth of		Active for chicks	References†
		S. fecalis R.	L. casei		
		mcg./ml.	*mcg./ml.*		
Pteroic acid	(0)	0.0008	0.07	(No)	(1)
Rhizopterin S.L.R. factor Formyl-pteroic acid	(0)	0.000034	Inactive	(No)	(2) (3) (4) (5)
Folic acid Pteroyl-glutamic acid Liver L. casei factor Vitamin B_c	(1)	0.003	0.00007	(Yes)	(6) (1) (7) (8) (9)
Pteroyl tri-glutamic acid Fermentation L. casei factor	(3)	0.0042	0.000061	(Yes)	(6) (7) (8) (10)
Pteroyl heptaglutamic acid Vitamin B_c conjugate	(7)	Slight*	Slight*	(Yes)	(11) (12)

* One microgram is equivalent to 0.003–0.006 mcg. of folic acid when measured with L. casei; 0.002 mcg. when measured with S. fecalis.

† References:

1. ANGIER, ET AL.: Science, **103**: 667 (1946).
2. KERESITESY, ET AL.: Science, **97**: 465 (1943).
3. STOKES, ET AL.: Science, **100**: 522 (1944).
4. RICKES, ET AL.: J. Amer. Chem. Soc., **69**: 2749 (1947).
5. WOLF, ET AL.: J. Amer. Chem. Soc., **69**: 2753 (1947).
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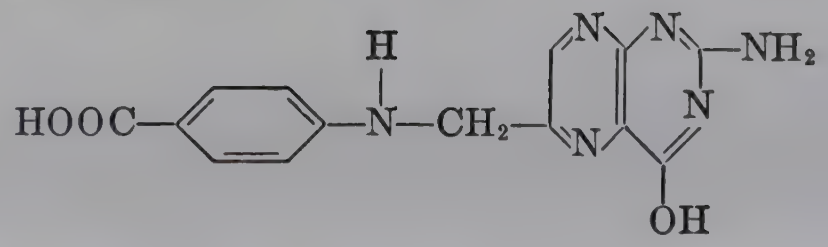
7. STOKSTAD, ET AL.: J. Am. Chem. Soc., **70**: 3,5 (1948).
8. HUTCHINGS, ET AL.: J. Amer. Chem. Soc. **70**: 1 (1948).
9. MOWAT, ET AL.: J. Amer. Chem. Soc., **70**: 14 (1948).
10. HUTCHINGS, ET AL.: Science, **99**: 371 (1944).
11. PFIFFNER, ET AL.: Science, **102**: 278 (1945).
12. PFIFFNER, ET AL.: J. Amer. Chem. Soc., **68**: 1392 (1946).

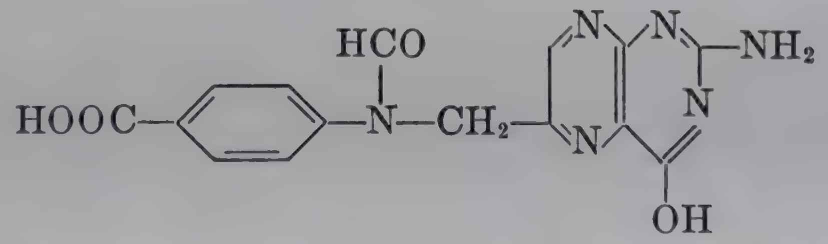
Pfiffner et al (7) describe their vitamin B_c crystals as follows:

"Pale yellow in color with no melting point but darken and char at about 250° C. Only slightly soluble in cold water (0.0016mg/ml at pH3 and 25° C) but soluble to

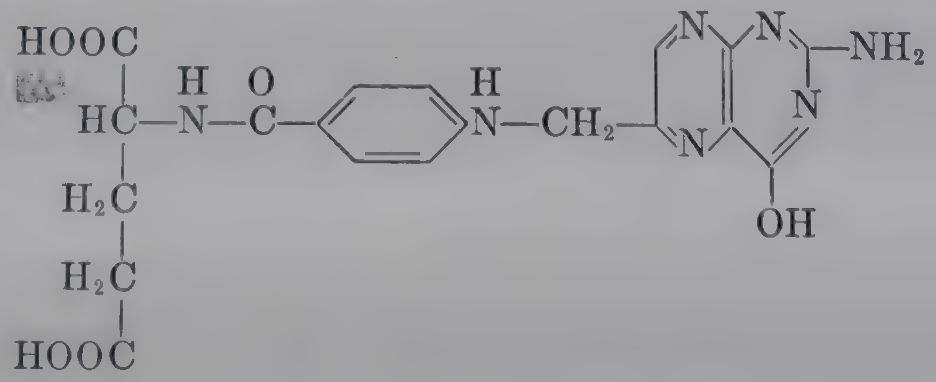
I. Pteroic Acid



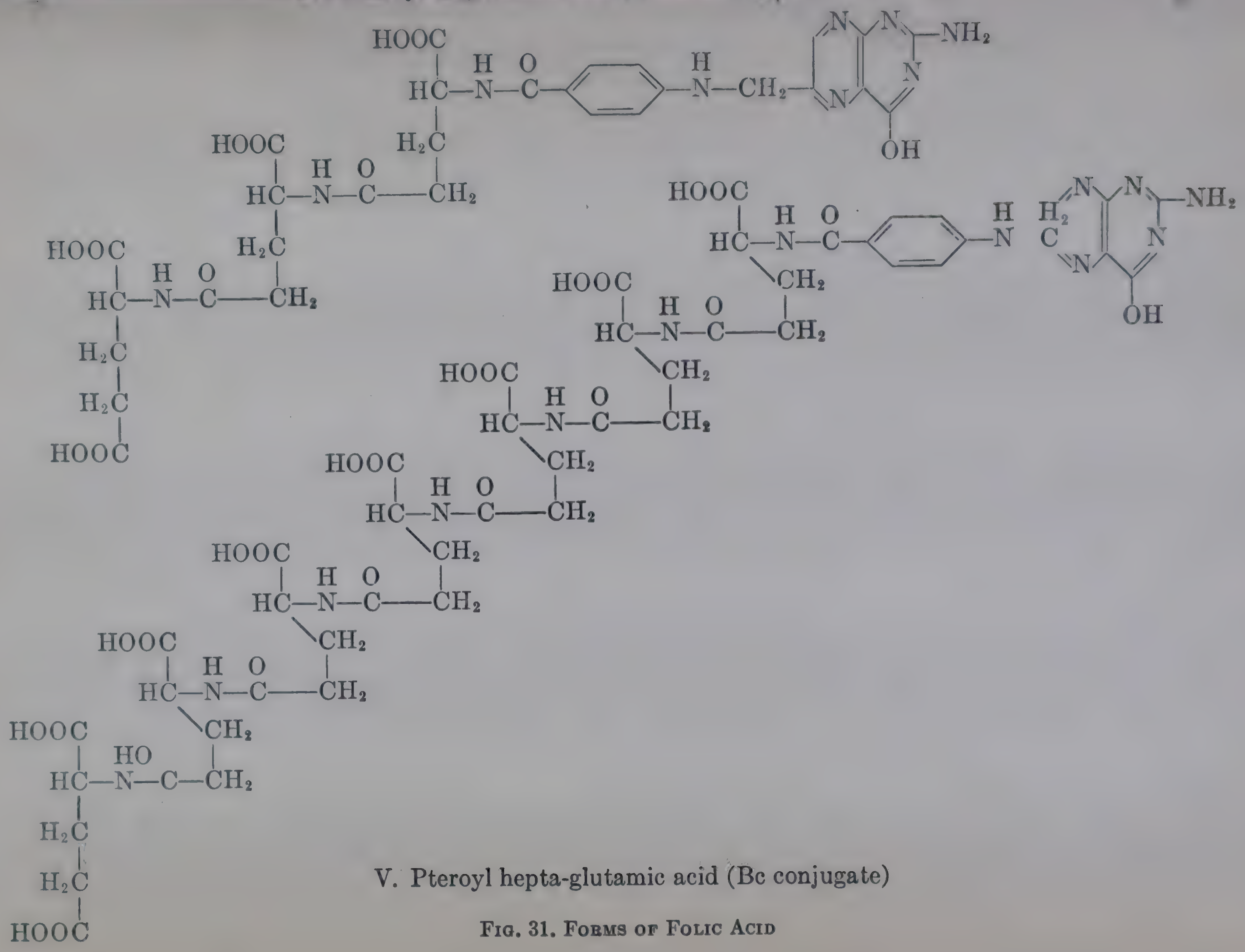
II. Rhizopterin or S. L. R. Factor



III. Pteroyl-glutamic acid (Folic Acid, Liver L. casei factor, Vitamin B_c



IV. Pteroyl triglutamic acid (Fermentati L. casei factor)



V. Pteroyl hepta-glutamic acid (Bc conjugate)

FIG. 31. FORMS OF FOLIC ACID

> about 0.1 percent in boiling water; slightly soluble in methanol and less so in ethanol and butanol; insoluble in acetone or chloroform but relatively soluble in acetic acid, phenol, and pyridine. The compound is both basic and acidic as shown by its ready solubility in water on addition of sodium, ammonium or barium hydroxide or on addition of mineral acids."

Stokstad et al (8) supply the following description of the crystals of L. casei factor which they isolated from liver:

> "At pH3 and 2° C their solubility in water was 10mcg./ml; at 100° C more than 0.5mg/ml. The extinction coefficients of a 0.1N NaOH solution were 565 at 255$\mu\mu$, 350 at 282$\mu\mu$; 195 at 365$\mu\mu$. The amount required per ml of culture medium for half maximum growth was 0.00007 mcg/ml for *L. casei*; 0.0003mcg/ml for *S. fecalis*.'

Pteroyl glutamic acid is essentially nontoxic. The lethal dose is reported as 125–600 mg. per kilo of body weight, depending upon the species of animal. This is 500 to 5,000 times the therapeutic doses that have been used successfully in the treatment of sprue and pernicious anemia.

Fermentation L. casei Factor (Pteroyl triglutamic acid)

In 1944 Hutchings et al (13) announced the isolation of a new *L. casei* factor; also active for *S. fecalis* and as a chick anti-anemia agent. When assayed with *L. casei* it was 85–90 per cent as active as PGA but only 6 per cent as active as PGA when assayed with *S. fecalis*. The product was named fermentation L. casei factor and it is produced in an aerobic culture of an unidentified organism belonging to the genus Corynebacterium. Its isolation and the method therefor was reported by Hutchings et al (14) in 1948.

Study of the degradation products (15, 16) of fermentation L. casei factor established the presence of 3 molecules of glutamic acid in the compound. Further data were obtained by the synthesis of Pteroyl-gamma-glutamyl-gammaglutamyl-glutamic acid which proved microbiologically identical with the isolated product. In this report, however, it was stated that the amount prepared was too small for more than microbiological comparisons.

Pteroyl heptaglutamic acid (Vitamin B_c conjugate)

In 1944 Binkley et al (17) reported that they had found in yeast a compound highly active for prevention of chick anemia but with little growth-promoting activity for either *L. casei* or *S. fecalis*. The product after enzymic digestion, however, yielded a solution highly active for *L. casei*.

This B_c conjugate was isolated in 1945 (18). It crystallized from a 5 per cent salt solution in the form of yellow, birefringent, microcrystalline spherulites, and on repeated crystallization as rosettes of microcrystalline needles. These crystals had no melting point but began to darken at about 200° C and partially melted at 230–260° C but never became entirely molten up to a temperature of 360° C.

The ultraviolet curves were almost identical with those of vitamin B_c but the molecular weight was much greater. From the E values and the separation of an ultraviolet transparent nitrogen moiety, the product appeared to be a combination of vitamin B_c (PGA) and amino acids, hence the original name of Vitamin B_c conjugate. The molecular size was 2.8 times that of B_c.

As stated above, while anti-anemic and utilizable by the chick, it had very slight activity for either *L. casei* or *S. fecalis;* one microgram equivalent to 0.003–0.006 mcg. of B_c when assayed by *L. casei,* 0.002 mcg. when assayed by *S. fecalis.*

There had been other reports (19, 20, 21, 22) of a form of folic acid microbiologically inactive but anti-anemic for animals. There were also reports of an enzyme capable of splitting the form into free folic acid (PGA). One such was prepared from chicken pancreas (22) and another from hog kidney (23). These so-called conjugases were widely distributed in tissues and Bird et al (24) have given the data shown in Table 51. Meanwhile, in 1946, Pfiffner et al (25) reported that the nitrogen moiety in vitamin B_c conjugate consisted to six molecules of 1(+)glutamic acid which were attached by peptide linkage, and that the conjugases that released B_c (PGA) from the conjugate were carboxypeptidases.

Further studies of the action of conjugase (26) showed that a given preparation of conjugase released more vitamin B_c in unit time from some substrates than from others. Crude yeast extracts were refractory to enzyme while purified concentrates were acted upon more readily. The explanation came with the discovery of conjugase inhibitors. (For discussion of the relation of these inhibitors to human utilization of B_c conjugate see Section III.)

Mims et al (27) state that a concentrate of inhibitor prepared from molasses was found to contain a large proportion of nucleic acid. Thymus and yeast nucleic acids were shown to be strong inhibitors of the conjugase from hog kidney, hog intestine, rat liver, and human leucocytes, but nucleic acid did not inhibit the purified conjugase from chicken pancreas. The inhibiting effect of thymus and yeast nucleic acid and of yeast extract was reduced by incubation with depolymerizing enzymes or by treatment with reducing agents. This effect of depolymerases is adduced as evidence that nucleic acid is the substance in yeast extract largely responsible for the in vitro inhibition of kidney conjugase by yeast extract.

TABLE 51

(From Bird et al. (24) 1945)

I. Effectiveness of different sources of enzyme in releasing folic acid from conjugate

Enzyme source	Enzyme preparation per cc. substrate	Substrate* dilution	Incubation time @ 37°	Free folic acid in enzyme (blank)	Free folic acid in substrate less blank
	grams		*hours*	*mcg.*	*mcg./ml.*
Control					1.8
Fresh beef heart	1.0	1:10	48	0.0	1.8
Fresh beef spleen	1.0	1:10	48	0.15	8.4
Clarase	0.5	1:20,000	48	4.15	8.7
Desiccated hog liver	0.08	1:10	72	0.55	2.6
Desiccated hog liver	0.20	1:10	72	1.50	9.0
Desiccated hog liver	0.50	1:10	72	3.25	13.0
Fresh hog liver	1.0	1:1000	48	3.5	16.4
Desiccated hog kidney	0.30	1:10	48	0.75	27.0
Desiccated hog intestine	0.20	1:10	72	0.10	26.3

* Substrate was a concentrate of folic acid conjugate from yeast.

II. Comparison of folic acid potencies obtained by L. casei assay before and after enzyme treatment

Substance number	Substance assayed	Enzyme method*	Folic acid per gram		Folic acid per gram chick assay
			Before enzyme	After enzyme	
			mcg.	*mcg.*	*mcg.*
96372	Water extract plasmolysed yeast	(1)	2.5	50	55
0742	Concentrate from yeast	(1)	11.6	198	200
38843	Yeast extract	(2)	2.0	52	50
38843	Yeast extract	(3)	2.0	57	50
42773	Bacto yeast extract	(1)	0.7	26	25
45903	Concentrate from yeast	(2)	12.6	250	280
58264	Liver extract	(1)	20.0	26	54
58264	Liver extract	(2)	20.0	52	54
65614	Concentrate liver extract	(2)	34.0	89	98
71124	Asparagus juice extract concentrate†	(4)	5.0	7	12
74914	Concentrate from yeast	(4)	48.0	7670	8135

* Methods: (1) desiccated hog kidney.
(2) extract of almond.
(3) extract of almond meal.
(4) extract of autolysed hog kidney.

† In the case of plant extracts no enzymatic procedure was found that would insure microbiological assay values equal to those obtained by chick assay.

These investigators (27) also reported the presence in the conjugase from hog kidney of an active sulfhydryl group. The conjugase is inhibited by sulfhydryl-combining reagents and the inhibition is reversed by cysteine and BAL. The presence of this sulfhydryl group may explain the loss of activity in attempts to purify the enzyme.

The relative inhibitory action of certain sources is shown in Table 52.

Rhizopterin or Formyl Pteroic Acid

In 1943 Keresztesy et al (28) announced in a brief note the discovery of a product which activated the growth of *S. Lactis R.* (now known as *S. fecalis*). It was inactive for *L. casei*. They called it temporarily the S.L.R. factor pending chemical identification. That identification was first reported (29, 30, 31) in 1947.

TABLE 52

Comparative inhibitor value of some natural substances

(After Mims et al. (27) 1947)

Source of inhibitor	Relative inhibitor activity*
Hammarsten's thymus nucleic acid	160
Levene's thymus nucleic acid	100
Yeast nucleic acid	79
Concentrate molasses inhibitor	60
Norit eluate from Type III yeast extract	32
Liver extract (70% alcohol soluble)	10
Type III yeast extract	7.6
Difco yeast extract	2

* Molasses equals 1.

The product got the name of rhizopterin because it was derived from the liquors remaining after fumaric acid fermentation by the organism Rhizopus nigricans and also because it contained the Pterin group. The compound was split by acid or alkaline hydrolysis into formic and pteroic acids. It contained no glutamic acid. However, Stokes et al (32) found that folic acid (PGA) was formed from S.L.R. factor when *S. fecalis* was grown in a medium free of folic acid but containing the S.L.R. factor. This was proven by the fact that when the whole culture, the cells, or the culture fluid was added in adequate amounts to folic acid-free media, they produced maximum growth and fermentation of *L. casei* and other folic acid requiring bacteria.

This result was interpreted to mean that the organisms converted S.L.R. factor into folic acid and would make Rhizopterin a true precursor of folic acid. Unlike xanthopterin, another folic acid precursor, it does not give rise to folic acid when incubated with rat liver suspensions.

Rhizopterin has the following properties:

A light yellow crystalline compound; insoluble in the common organic solvents and water, but soluble in mineral acids and in alkalies. At pH 11 Rhizopterin showed E maxima of 940 at 255 $\mu\mu$; 219 at 365 $\mu\mu$. At pH 7, E maxima of 550 at 245–275 $\mu\mu$ and 173 at 350 $\mu\mu$. At pH 3, E maxima of 612 at 270 $\mu\mu$ and 155 at 345 $\mu\mu$. At pH 1 E maxima of 614 at 252.5 $\mu\mu$ and 233 at 325 $\mu\mu$.

Titration of rhizopterin was carried out by dissolving in 0.1N alkali and treating with standard acid; precipitation occurred at pH 7, mid point of curve at approximately pH 7.2.

Rhizopterin has been synthesized by treatment of Pteroic acid with formic acid. The synthetic product showed the same microbiological activity as the isolated rhizopterin, viz., half maximum growth of S. fecalis at 0.000034 mcg. /ml; essentially inactive for *L. casei.*

Rhizopterin is inactive for hemoglobin formation or for growth promotion of chicks. It did not cure the granulocytopenia induced by folic acid deficiency in rats.

Its descriptive chemical name is:

p-N-2-amino-4-hydroxy pyrimido 4,5-b pyrazin-6-ylmethyl
-formamido benzoic acid.

Significance of the Formyl Group in Rhizopterin

Shive et al (33) produced a formyl derivative of folic acid (Formyl Pteroyl glutamic acid) and have suggested that the formyl group may play a role in the synthesis of purines, and may serve to bring about the introduction of a single carbon atom.

Pteroic Acid

Waller et al (9) have reported the synthesis of Pteroic acid. It was not active as an antianemic factor for chicks but stimulated the growth of both *S. fecalis* and *L. casei* (See Table 50).

Distribution of Folic acid in Foodstuffs

As previously stated, folic acid is present in many foods partly as the free folic acid (PGA), but predominantly in conjugated form. Assay therefore requires the breakdown of the conjugate to get response to microorganisms, the use of some type of conjugase. Olson (35) has reported some data on this distribution part of which is summarized in Table 53.

They list fresh deep green leafy vegetables and liver as very rich in folic acid; fresh green vegetables, cauliflower and kidney as rich sources; beef, veal and dry wheat cereals intermediate in richness; and root vegetables, tomatoes, cucumbers, light green leafy vegetables, bananas, pork, ham, lamb, cheese, milk, dry rice, corn breakfast cereals and many canned foods as low in the vitamin.

Losses are large when stored at room temperature but normal refrigerator temperatures are fairly effective in the retention of potency.

TABLE 53

Distribution of folic acid in foodstuffs

(After Olson et al. (35) 1947)

I. Evidence of presence of folic acid as conjugate

Foodstuff	Relative assay values by water extract		Takadiastase		Kidney conjugate	
	S. fecalis	L. casei	S. fecalis	L. casei	S. fecalis	L. casei
New Zealand spinach	90	95	180	150	170	155
Chard	71	76	86	87	123	123
Cucumbers	2.3	2.6	5.0	6.7	6.5	12.5
Broccoli	58	80	54	83	90	110
Tomatoes	13	11	12	12	14	12

II. Average distribution in mcg. per 100 gms. fresh material

Food	Assayed by		Food	Assayed by	
	S. fecalis	L. casei		S. fecalis	L. casei
Spinach	82	127	Cucumbers	5	7
Chard	100	111	Carrots	11	13
New Zealand spinach	185	170	Potatoes	5	
Parsley	88		White radishes	10	11
Green asparagus	48	44	Wheat Bkst cereal	29	
Broccoli	54	83	Wheat Bkst cereal	46	
Cauliflower	14	54	Wheat Bkst cereal	19	
Peas	59	50	Corn Bkst cereal	6	
Green snap beans	51	41	Rice Bkst cereal	11	
Leaf lettuce	8	16	Cheddar cheese	5	
Cabbage	12		Green bananas	13	21
Kohlrabi	7		Ripe bananas	7	15
Tomatoes	10	11	Very ripe bananas	4	8
			Apples	1	

mcg. equals micrograns.

Xanthopterin

As early as 1937 Tschesche and Wolf (36) reported that rats made anemic by feeding goat's milk responded with reticulocyte and red cell increase to administration of Xanthopterin. (For structure see figure 32.) Koshara et al (37) failed to confirm this finding. This conflict of findings concerning xanthopterin has continued. In 1941 Simmons et al (38) reported it corrective of an anemia in fish produced by feeding a high protein diet plus yeast. In 1943 Totter and Day (39) reported that xanthopterin was partially effective in alleviating nutritional cytopenia in the monkey and capable of replacing folic acid in rats fed succinylthiazole.

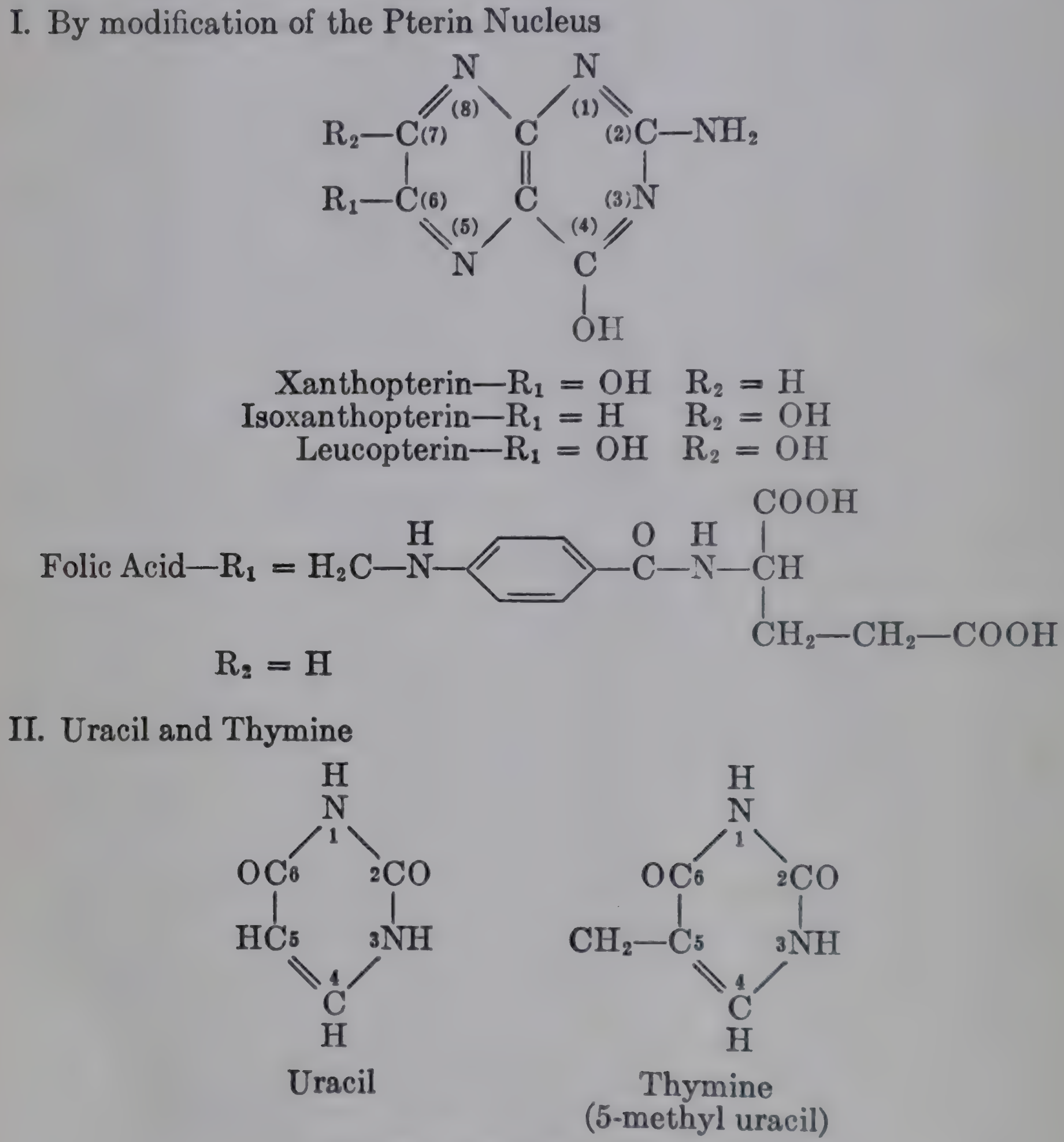

I. By modification of the Pterin Nucleus
Xanthopterin—R₁ = OH R₂ = H
Isoxanthopterin—R₁ = H R₂ = OH
Leucopterin—R₁ = OH R₂ = OH
Folic Acid—R₁ =
R₂ = H
II. Uracil and Thymine
Uracil
Thymine
(5-methyl uracil)

Fig. 32. Folic Acid Precursors

The effect on rats was not reproducible by Daft and Sebrell (40) or by Axelrod et al (41), and O'Dell and Hogan (42) found it inactive for chick anemia.

However, Wright and Welch (43) obtained evidence that fresh rat liver is capable of synthesizing folic acid from xanthopterin. Totter, Mims and

Day (41) checked this finding by using livers from two vitamin M deficient monkeys. They reported that the livers from these two monkeys were strikingly low in preformed folic acid, and that extra folic acid was produced from xanthopterin by the liver of the monkey not receiving the pterin, while liver of the other animal produced extra folic acid from yeast and from xanthopterin when yeast was also added. Chicken liver produced no folic acid from xanthopterin alone, but did so in the presence of yeast and also from yeast alone. These studies of the possible relation of xanthopterin as a precursor of folic acid are principally interesting today as one of the things that led to the ultimate chemical identification of folic acid (6).

The relation of xanthopterin to folic acid as a hematopoietic factor has been further studied by Norris and Majnarich (55). They have used bone marrow cultures and also weanling rats made anemic on a synthetic diet plus 1 per cent sulfathiazole for test purposes.

In their first report (55a) they record supplementation of the bone marrow cultures with xanthopterin, folic acid (PGA), and normal blood serum. They recorded red and white cell counts and also reticulocyte counts. They got striking increases in these counts with 5 mcg/ml of xanthopterin and with 0.1 ml of normal blood serum but not with 5 mcg/ml of folic acid.

In their weanling rat tests (55b) at 22 days on the diet the red cell count was 3.4 × 10^6/cu mm. and the hemoglobin 6.8 gms/100 ml. At that time these rats got 100 mcg of xanthopterin per day for five days by injection and as a result the red cell count rose to 9 × 10^6/cu mm and the hemoglobin to 15 gms/100 ml in the next 20 to 30 days. With the injection of the same amount of PGA there was a response similar in magnitude but with a lag in time. This they interpret as due to time for conversion of the folic acid to xanthopterin.

In the third paper (55c) they reported study of effect of variations in amount of xanthopterin in curing the rat anemia. 1 mg/kilo of body weight produced spectacular rise in cell counts, hemoglobin and hematocrit which increased regularly for 10 to 15 days following injection but 5 mcg/kilo was less effective and 10–20 mcg/kilo without favorable effect indicating that there is an optimum intake for xanthopterin as was also indicated in the bone marrow culture studies.

In this comparative series folic acid in an amount equal to the optimum xanthopterin dose was less effective. They therefore raise the question: "Is xanthopterin an active form of folic acid?"

Still more recently (53) they have reported discovery of a B_{14} vitamin apparently formed from xanthopterin by enzyme action that is 5,000,000 times as active as xanthopterin in their bone marrow cultures. For discussion of this factor see Chapter XIX.

Thymine

In 1944 Stokes (45) suggested that one of the compounds synthesized for blood cell formation might be a derivative of thymine. He found that certain bacteria in the presence of thymine no longer required folic acid but a much larger amount of thymine than of folic acid was necessary to meet requirement. Both Stokes (45) and Lampen and Jones (46) suggested that folic acid functions as a prosthetic group in an enzyme concerned with the synthesis of thymine or related compounds. In 1945 Hutchings et al (47) reported a study of the effect of various pyrimidines, including thymine, on the growth of *L. casei*. Thymine (See fig. 32) is 5-methyl uracil. Among 5-substituted uracils they found a number of compounds that actually inhibited the growth of *L. casei*, e.g. 5-hydroxy uracil and 5-amino uracil. Growth could be restored by increasing the concentration of either thymine or folic acid.

However these investigators disagree with Stokes' hypothesis as an adequate explanation of the action of thymine because of the behavior of 5-halogen pyrimidines and 5-nitro uracil. Brom-uracil, for example, completely inhibited the growth of *L. casei* with thymine as the nutrient, but had little or no effect when folic acid was used as the nutrient.

These results seemed to indicate that the growth of *L. casei* with folic acid as a nutrient did not depend upon an intermediary synthesis of thymine. 5-nitro uracil prevented the growth of L. casei with folic acid at concentrations which had little or no effect on the growth with thymine. These investigators prefer to believe that the two nutrients, thymine and folic acid, act as alternatives rather than as two components of an anabolic system.

That massive doses of thymine can at least partially substitute for folic acid in the treatment of nutritional macrocytic anemia, pernicious anemia, and sprue has been demonstrated by Frommeyer and Spies and coworkers (48). They reported that the minimum amounts for effect were 15 grams orally of thymine to get the effect of 10–20 mg. of folic acid; or 0.5 ml. of liver extract intramuscularly. They suggest that thymine may be converted into folic acid in the intestinal tract.

Folic Acid Antagonists

Recent studies of the action of sulfonamides indicate that their function is to inhibit growth of microorganisms by competitive interference with folic acid synthesis by the bacteria by way of Paba (49). But other antagonists of folic acid have been found.

In 1947 Martin et al (50) reported synthesis of d(−)methyl folic acid as a *displacing* agent for folic acid, the effective ratio of the inhibitor to the metabolite being 150:1 for *S. fecalis*.

Another antagonist which apparently differs from that of Martin in that the glutamic acid is the natural (1) form was prepared by Seeger, Hultquist and Smith (51), and its effect on rats was investigated by Franklin et al (52). The crude compound was not purified, but was shown to inhibit the growth promoting effect of folic acid on both *L. casei* and *S. fecalis*; minimum ratio 20–30 for *S. fecalis*; 1000 for *L. casei*. The inhibition was reversible by folic acid (PGA). The effect of the crude antagonist on rats was similar to the effects of vitamin M deficiency in the monkey. And this antagonist also manifested action in mice and in chicks.

Swenseid et al (53) have reported a study of several compounds analogous to PGA. Of these, 4-amino folic acid was an efficient inhibitor, and 2, 4 diamino-6, 7 diphenyl pteridine had considerable effect.

Hutchings et al (54) have reported that pteroyl aspartic acid acts as an antagonist in the chick but not in the rat. There is considerable evidence that for vitamin activity there must be at least one glutamic acid residue attached to the pteridin fraction. Pteroic acid and Rhizopterin, for example, lack the glutamic acid residue and are inactive for *L. casei* and for man. Introduction of a 7-methyl group or substitution of an amino group for the 4-hydroxy group in Pteroyl glutamic acid produces an antagonist.

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Section III. Evaluation of Folic Acids in Human and Domestic Animal Nutrition

There have been several excellent summaries of the clinical experience with the various folic acids (1, 2, 3). It has now been demonstrated that Pteroyl glutamic acid (PGA), Pteroyl triglutamic acid (P3GA) and Pteroyl heptaglutamic acid (P7GA) are all active hematopoietically for man. The hematological remissions brought about by PGA in patients with pernicious anemia resemble those produced by liver extracts, but continuous administration to patients with pernicious anemia does not protect them from disabling neurological complications known as subacute combined sclerosis of the cord or combined system disease (4, 5, 6).

It is therefore established that the liver extract antipernicious anemia is not chemically identical with any of the folic acids yet isolated. It is possible that the newly discovered vitamin B_{12} may be the liver factor, but its chemical nature has not yet been determined other than that it is a cobalt complex of some sort. (See Chapter XIX)

The only controversial point about the utilization of the three folic acids by man has concerned the ability of the pernicious anemia patient to utilize Pteroyl hepta glutamic acid (Vitamin B_c conjugate). Originally it was suggested that the pernicious anemia patient was unable to break down the conjugated forms of folic acid to free folic acid (PGA). But additional studies have made this viewpoint untenable, and do account for variation in the response of different patients. Bethell et al (7) have made a special study of the metabolic function of folic acid and folic acid conjugate in patients with macrocytic anemia.

Their explanation of variation in response to the conjugate as it occurs in various sources is that it depends on the presence or absence of conjugase inhibitors. They administered Pteroyl glutamate and the conjugate orally to a group of 16 patients. The series included 9 patients with pernicious anemia in relapse, two with macrocytic anemia following gastrectomy, one with chronic liver disease associated with macrocytic anemia, one with nutritional macrocytic anemia, and three with pernicious anemia in liver extract remission. Hematopoietic response was observed and the urinary excretion of folic acid (PGA) measured during the administration of both the conjugated and free vitamin.

In general it was found that ability to utilize the conjugate and convert it to free folic acid depended upon the amount of conjugase inhibiting substances present. In the absence of inhibitors the conjugate was utilized. (For inhibitor distribution see Table 51.)

These same investigators, in a second report dealing with normal persons, found further evidence of the importance of conjugase inhibitors. When a concentrate of the folic acid conjugate free of inhibitors was administered, the urinary excretion of the released folic acid was comparable to that obtained when an equivalent amount of the vitamin was given in the free form, 1266–1288 mcg. on consecutive days. But administration of conjugate concentrates containing conjugase inhibitors resulted in lower PGA excretions, 153–244 mcg. on consecutive days, values definitely lower than when the free folic acid was given or the conjugate without inhibitors.

The pteroylglutamates are today recognized as required by the monkey, chick, turkey, fox, mink, dog, the sulfa-treated rat, guinea pig and mosquito and certain microorganisms. An experimental deficiency of folic acid has not been produced in man but in view of the undoubted activity of the vitamin in sprue, in nutritional macrocytic anemia, and in pernicious and related anemias, it seems probable that it is a definite human requirement.

A deficiency of the factor in the higher forms produces an anemia and a leucopenia of varying intensity. Thrombocytopenia may also result, and the deficient animals usually exhibit weight loss. In some species oral lesions and diarrhea appear. From the effect in sprue cases it appears that the vitamin is not only of value in correcting and preventing hematopoietic effects but also in improving gastrointestinal absorption (8, 9). Goldsmith (10) and Kemp (11) have obtained good responses in cases of nutritional macrocytic anemia which are usually associated with poor dietary habits.

Folic Acid and Domestic Animals

Ruminants: Cattle and sheep apparently need no folic acid supplement and that appears generally true of all domestic animals on usual rations, due partly to bacterial synthesis and partly to distribution of the factor in natural feedstuffs. That does not mean, however, that folic acid is not a requirement of animals as well as man.

By inducing folic acid deficiency in chicks, turkeys and in swine, the effect of deficiency when it occurs has been established, and in these three types of domestic animals we have data on the effect of such deficiency if it should occur.

Fowls: It was the effect of folic acid deficiency in chicks that led to Hogan and Parrott's (12) postulation of vitamin B_c which in turn resulted in its chemical identification and proven identity with Liver L. casei factor.

Campbell et al (13) conducted studies with chicks using crystalline vitamin B_c (PGA) and have described in detail the changes that occur in the blood picture during a prolonged B_c deficiency. They used three groups of white Leghorn chicks. The first group received a normal diet consisting of a commercial broiler ration containing 18 per cent protein. The second group got a purified diet containing all the known vitamins except B_c. The third group got the same diet as group two plus varying amounts of crystalline vitamin B_c.

The group on the normal diet grew well. The blood picture remained fairly constant during a twenty eight day test. The erythrocytes in the blood reached full maturity by the end of two to three weeks. In the first two weeks there was a moderate polychromasia and basophilia demonstrated in the nucleated erythrocytes. The one day old chicks showed granulations of the leucocytes but this disappeared after a few days.

Apparently the normal diet resulted in no manifestation of macrocytic anemia and the Committee on Animal Nutrition (14) in listing the essential ingredients for the diet of starting chicks and of laying and breeding hens does not include folic acid in their recommended vitamin supplement list.

The second group on the B_c-deficient synthetic diet developed a characteristic syndrome. After one or two weeks the birds became lethargic and the wings drooped. After two to three weeks symptoms of incomplete feathering developed, and after 4 to 5 weeks the enamel on the feather shaft gradually disappeared. Mortality amounted to about 50 per cent.

As to the blood picture, it remained essentially similar to that of the birds of group one for the first week but thereafter the erythrocyte count dropped steadily from 2 million per cubic mm to in one case as low as 300,000. Immature red cells appeared showing basophilia. In the second week there was distinct macrocytosis, and after three weeks the normoblasts, pronormoblasts, and myeloblasts showing mitotic figures appeared in the blood. In the last stages a pronounced abnormality in the size of the cells (anisocytosis) developed.

The leucocyte picture did not become abnormal until the second week. Disappearance of granulation of leucocytes took place in the first days in the same manner as in the normal group of chicks, but during the second week a pronounced leucopenia developed, affecting all types except the heterophiles whose count remained unaltered. These later showed a distinct shrinking (pyknosis) and the leucocytes became vacuolated. Also during the second week, the thrombocytes showed dissolution of the chromatin of the nucleus (karyorrhexis), shrinking (pyknosis) and later cytoplasmic vacuoles. The latter change became so pronounced in the third or fourth week that the thrombocytes resembled lymphocytes and varied greatly in size and shape, but thrombocytopenia did not occur.

Variation of B_c dosage in the third group indicated to these investigators that 100 mcg. of B_c per 100 grams of diet was sufficient for normality; less (40 mcg.) for maintaining normal red cell volume and thrombocyte count than for red cell and leucocyte count.

Angier et al (15) have reported that 50 mcg. of Liver L. casei factor per 100 grams of ration produced excellent response in chicks. They grew as rapidly and maintained as high a hemoglobin level as did chicks on a diet of natural feedstuffs. Briggs et al (16) got normal results in chicks with a concentrate of the spinach folic acid at 50 mcg. per 100 grams of ration.

It has also been reported (17) that folic acid deficiency in chicks may result in impairment of response to estrogens such as stilbestrol and also (18) in decreased resistance to malarial infection.

There have been a number of reports on the effect of folic acid deficiency in turkey poults. Jukes et al (19) found that, in addition to slow growth and hematological changes similar to those in chicks, the turkeys developed a cervical paralysis. They put the requirement at 0.8 mg. per kilo of ration.

Russell et al (20) put the requirement for normal growth at 2.0 mg. kilo of feed when the feed is given at 25 or 43 per cent protein levels. They got only subnormal feathering when on this amount of folic acid at 25 per cent protein level but normal feathering with 2.0 mg. per 100 grams of ration at 43 per cent protein level.

Swine: Cartwright et al (21) have reported the production of folic acid deficiency in swine. To induce this deficiency they used both a folic acid antagonist and sulfasuxadine. The antagonist was a crude methyl folic acid preparation. With this treatment the animals became listless, weak and ate poorly. Hair loss was not extensive, but the hair became thin and lustreless. Moderately severe diarrhea was present.

The pigs became markedly anemic in twenty-one to forty-two days. Small doses of folic acid (PGA) rapidly relieved the anemia; partially in one pig by liver extract and not at all by protein therapy. But, only by protein therapy was the leucopenia and neutropenia relieved. They also claim that liver anti-pernicious anemia factor cannot replace folic acid for the pig. Cunha et al (22) also reports that folic acid was more effective for pigs than the anti-pernicious anemia factor of liver.

In an earlier report Cunha et al had reported that addition of folic acid alone or Paba alone or in combination with inositol or biotin had no benefit in pigs in the matter of external appearance or feed utilization and only a small stimulation of hemoglobin formation.

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CHAPTER XVII. ASCORBIC ACID OR VITAMIN C

Section I. Functions of Ascorbic Acid

The discovery that lemon juice and lime juice contained a factor curative and preventive of scurvy dates back to the 1700's (1). In 1804 Sir Gilbert Blaine secured regulations enforcing supply of lime juice to sailors of the British Navy, and in 1865 similar regulations were adopted for the British Mercantile Marine hence the name "lime juicers". Funk (2) in 1914 first suggested that scurvy was a vitamin deficiency disease but Holst and Fröhlich (3) in 1907 instituted modern reasearch for identification of the antiscorbutic factor by producing the disease in guinea pigs and Höjer (4) greatly extended the use of this animal for the study of experimental scurvy.

The actual chemical identification of the vitamin is credited to Szent-Györgyi (5), though King and Waugh (6) had already obtained from lemon juice an active antiscorbutic concentrate. The chemical structure was established in 1934 by Haworth, Hirst and coworkers (7) and the vitamin was synthesized by Reichstein et al (8) in 1933–34.

Scurvy

The prime interest in ascorbic acid or vitamin C was in its value as a scurvy preventive and cure. Also, since hemorrhage is the most characteristic symptom of scurvy, study was directed as to how the vitamin prevents bleeding. With the isolation and availability of the vitamin in the pure form, its reversible oxidizability suggested still other functions in metabolism.

That ascorbic acid is essential to the prevention of scurvy has been fully established, but Dalldorf (8) has pointed out that though lack of the vitamin is a specific cause of the disease, there may be other factors controlling its development and that this accounts for occasional discrepancies in the response of individuals to vitamin C therapy. Elmby and Warburg (10) for example, have reported that of 29 cases of mild scurvy 26 responded within ten days to 300 mg. of ascorbic acid given orally, but three showed no improvement and still failed to respond to 300 mg. given parenterally. They did, however, respond to the juice of ten lemons given orally, suggesting that lemon juice contains some other factor or factors than ascorbic acid that may play a role in scurvy protection. (See p. 323).

In 1919 Aschoff and Koch (11) advanced the view that scorbutic hemorrhages and other tissue changes of scurvy were due to a lack of something essential to the behavior of intercellular materials, especially those associated with the connective or mesenchymal tissues. Dalldorf (9) pointed out that the primary morphologic effect of ascorbic acid deficiency does appear in the intercellular substances of certain mesenchymal tissues. In 1926 Wohlbach and Howe (12) advanced a theory concerning ascorbic acid function.

If we consider loose connective tissue, under normal conditions the type cell (fibroblast) lies in an amorphous ground substance within which fibrils are formed, which, in turn, become cemented together into wavy bands of collagen. In guinea pigs depleted of ascorbic acid, the fibroblasts are present just as in healthy pigs, but the fibrils and collagen fail to form. Within 18 hours after adequate ascorbic acid dosage these intercellular substances appear.

In bone, the functioning cells are osteoblasts and the intercellular material is osteoid tissue. In the teeth the functioning cells are the odontoblasts and the intercellular substance is dentin. In both bones and teeth lack of adequate ascorbic acid results in a change in character of these intercellular materials and they are restored to normality by ascorbic acid administration.

There is, then, general agreement that lack of ascorbic acid affects the formation of normal intercellular substance and bleeding is explained on the basis that in ascorbic acid deficiency the intercellular substance in the walls of the capillaries is faulty and makes the capillaries leak blood. The controversial point has been: whether ascorbic acid acts by supplying something the cells need for manufacture and control of intercellular substance or whether it produces its effect by interference with the metabolism of the fibroblasts, osteoblasts, and odontoblasts themselves.

Fish and Harris (13) and Höjer (4) are among those who took the view that lack of ascorbic acid produces atrophy of the cells themselves. Wohlbach and Howe (12) held that ascorbic acid is a building material for the intercellular substance.

Capillary Bleeding and Intercellular Substance

In 1920 Hess (14) reported that applying a tourniquet and thus subjecting capillaries to increased blood pressure produced petechial hemorrhages or blood clot spots that were visible just under the skin in scorbutic individuals. Göthlin (15) developed a test for ascorbic acid deficiency based on this phenomenon and Dalldorf (16) perfected a capillary resistance manometer for the purpose.

The endothelium, or lining membrane of the capillaries, is believed to be fused together by a cementing substance. The capillary is also surrounded by connective tissue and the endothelium is ensheathed with collagenous fiber. It is not yet clearly established whether it is the endothelium fusing material that is lacking in scurvy or failure of the connective tissue cells to form collagen. Dalldorf (16) inclines to the view that it is the intercellular substance that is lacking and not loss of ability of the connective tissue cells to proliferate the leak-preventing substance. In other words, he agrees with the Wohlbach-Howe theory rather than with the cell atrophy theory.

Scorbutic Changes in Bone

Scurvy often produces lesions at the costa-chondral junction and at the end of certain bones. At these regions there is often a cessation of bone formation and replacement with collagen-poor connective tissue in which may be embedded fragments of densely calcified cartilage. The cells in the regions are frequently osteoblasts which have reverted to primitive fibroblasts. The condition suggests that in the absence of ascorbic acid the osteoblasts, being unable to form osteoid tissue, revert to their primitive connective tissue form and try to set up a fibrous union. The zone where this occurs is spoken of as the "gerüstmark" or marrow framework. This "gerüstmark" shows up in the X-ray and is one means of diagnosing ascorbic acid deficiency. Such bone changes are also usually accompanied by periosteal hemorrhages.

Some of these changes in bones are strikingly similar to those that occur in rickets (vitamin D deficiency), and Schwachman and Gould (17) have reported that ascorbic acid stimulates production of serum phosphatase, and deficiency lowers its production. Dalldorf has also called attention to the fact that stress modifies the site of the bone lesions and the extent and involvement of the various structures.

Scorbutic Anemia

There has been considerable discussion in medical papers as to whether the hypochromic anemia that sometimes accompanies manifestations of scurvy is a specific result of ascorbic acid deficiency or correctible with ascorbic acid administration. In 1941 Ralli et al (18) concluded that the absence of ascorbic acid could not be considered solely responsible for this anemia. Of the 53 case studies reported by McMillan and Ingalls (19) in 1944, 40 cases had slight to moderate anemia. The degree of anemia failed to correlate with severity of hemorrhage, plasma ascorbic acid content, or saturation requirement, and 11 subjects receiving saturation doses of ascorbic acid plus 100 mg. daily maintenance doses showed no more rapid blood regeneration or greater reticulocyte response than 4 controls on the hospital diet alone. The hospital diet supplies 15 mg. of ascorbic acid and 14 mg. of iron daily. They expressed the view that the anemia was of nutritional origin but not due to lack of ascorbic acid alone.

This viewpoint was further strengthened by the studies of Crandon, Lund and Dill (20) who in 1940 reported on a single individual whose sole diet deficiency was in ascorbic acid. This individual failed at any time during his manifestations of scurvy symptoms to develop an anemia.

In contrast to these findings Vilter, Woolford and Spies (21) in 1946 described 19 cases of scurvy (11 in detail) encountered in the Cincinnati General Hospital. They found moderately severe anemia present in 17 of the 19 subjects. Because they got corrective response in these cases to ascorbic acid administration they believe it was at least one factor in the anemia.

Scorbutic Tooth and Gum Changes

In the teeth, the dentin filling the space between the enamel or cement layer and the root canal is the product of specialized cells called the odontoblasts. In the guinea pig it has been shown that deprivation or inadequate supply of ascorbic acid produces these changes: within four or five days the odontoblasts shorten and become separated from the dentin by a fluid zone. If deprivation of ascorbic acid is complete and is maintained until the death of the animal (usually in about three weeks) these odontoblasts revert to a spindle form and are indistinguishable from the connective tissue cells in the pulp of the tooth. Simultaneously the Tomes canals widen appreciably. bringing about a porosity in the dentin. The teeth also cease to grow.

If ascorbic acid deficiency is only partial and prolonged for several months, the odontoblasts continue to secrete but produce, instead of dentin, a substance resembling bone which gradually fills the pulp canal. Addition of adequate ascorbic acid brings a prompt return to original appearance and function of the cells affected by the deficiency. These scorbutic dentin changes are usually accompanied by tendency to hemorrhage. The dental lesions begin at the crown of the tooth and proceed toward the root.

Ascorbic acid deficiency also affects the gums. It may be a cause of ginigivitis, gum hemorrhage, and a certain type of pyorrhea (periodontoclasia). Boyle, Bessy and Wohlbach (22) have pointed out that in ascorbic acid deficiency there may be changes in the soft and calcified tissues around the tooth. They suggest that there are two types of pyorrhea: a local inflammatory disease and a systemic process causing a diffuse atrophy of the alveolar bone. The latter may be a consequence of ascorbic acid deficiency. In support of that view they cite 23 cases improved by daily doses (150–200 mg.) of ascorbic acid.

Farmer and Abt (23) state:

> "Although there is still a dearth of exact knowledge of vitamin C in its relation to dental and gingival disease in man, there is general unanimity of opinion that an adequate intake of vitamin C is necessary for normal tooth growth and tooth structure, and the maintenance of healthy gums in man."

Ascorbic Acid and Wound Healing

One of the reasons for incorporation of maximal content of ascorbic acid (75 mg. daily) in the rations of the Armed Forces of World War II was recognition of its value in wound healing. As early as 1937 Lanman and Ingalls (24) noted poor repair and improper collagen formation in experimental wounds in guinea pigs. Crandon and Lund (25) and others have stressed the importance of ascorbic acid dosage preoperatively to insure postoperative healing and reduction of the danger of hemorrhages.

Bartlett et al (26) reported an extensive investigation of the effect of ascorbic acid on experimental wounds in test animals. They checked both the distribution of the vitamin in the wound tissues and the resistance of the scar tissue to pressure.

In the first series of ten animals on ascorbic acid-deficient diet the average control tissue content was 0.25 mg. per 100 gms. after operation 0.29 mg./100 gms. In contrast animals on high vitamin C diet showed a mobilization of the vitamin in the healing scar tissue; 6.53 mg./100 gms. tissue compared with biopsy controls of 1.57 mg./100 gms. The adjacent area also showed mobilization; 3.13 mg./100 gms. and the distant wall 2.7 mg./100 gms.

In the second series tensile strength of the scar tissue was measured by inflating the peritoneal cavity with air (10 days after operation) and recording the pressure necessary to burst the wound. The average pressure required in scorbutic pigs was 70 mm. of mercury in contrast to 140 mm. for the saturated animals; for the latter the range was from 127–287 mm. for complete rupture.

Ascorbic Acid and Country Rheumatism

There is strong evidence that what used to be called country rheumatism, namely stiff joints that developed during long winters on diets lacking green stuffs, was actually a form of mild scurvy; the stiffness due to joint hemorrhages that cleared up when diets were changed with the arrival of Spring greens.

Ascorbic Acid and Liver Damage

Murakami (27) demonstrated that when guinea pigs were fed a diet deficient in ascorbic acid their livers detoxified less well and excreted smaller amounts of bile and bile pigments. This condition was reversed by ascorbic acid administration. Ratsimamanga (28) claimed that liver glycogen was also reduced in scorbutic guinea pigs. Beyer (29) found the hepatic toxic effect of hydrazine reduced by ascorbic acid and enhanced by ascorbic acid deficiency. The mechanism of the action has not been explained, but it has been suggested that the ascorbic acid may exert its effect indirectly by permitting normal metabolism of carbohydrate and protein.

Ascorbic Acid and the Adrenal Cortex Hormone

"Addison's Disease" is the name given to disease of the supra-renal capsules. A pigmented (bronzed) skin is one characteristic of the disease and several observers (30) (31) (32) (33) have reported reduction of the pigmentation by administration of ascorbic acid. Schroeder (31) showed that it was able to inhibit the pigment formation from dopa by slices of guinea pig ear.

The adrenals are known to have a high content of vitamin C in both the cortex and the medulla and the usual treatment of Addison's disease is administration of adrenal cortex extract.

In 1940 Giroud et al (33) reported that synthesis of the adrenal cortex hormone is dependent upon the presence of ascorbic acid in the adrenals, and Sayers et al (34) have presented confirmatory evidence. In 1946 Sayers et al (35) found that the injection of adrenocorticotropic hormone into rats and guinea pigs produced a prompt fall in adrenal ascorbic acid and also a decrease of cholesterol. From these results the authors suggested that cholesterol is a possible precursor of the adrenal cortical steroids, and that the release of these steroid hormones has a special relation to the decrease in adrenal content. Li and Evans (36) suggest that the lowering of ascorbic acid in the adrenal caused by adrenocorticotropic hormone stimulation may possibly be due to increment in the ascorbic acid-steroid complex secretion. Lowenstein and Zwemmer (37) have reported the isolation of a new steroid from aqueous extracts of the adrenal glands. This compound contained a molecule of ascorbic acid and affected carbohydrate metabolism in a manner resembling response to dehydrocorticosterone.

Ascorbic Acid and Resistance to Infection

Claims have been made that ascorbic acid increases resistance to infection and is beneficial in reducing development of the common cold.

In 1942 Glazebrook and Thompson (38) reported a study of a group of boys in an English institution where food handling had reduced the ascorbic acid intake to only 15 mg. per day per boy. Part of this group was given an ascorbic acid supplement and while in a six month's period there was no difference in the incidence of colds, the boys in the treated group spent only 2.5 days in the infirmary compared with 5 days for the untreated group. Glazebrook however emphasizes that the results should not be interpreted to mean that vitamin C plays a major role in resistance to disease.

Sigal and King (39) demonstrated that ascorbic acid protected guinea pigs from the effects of diphtheria toxin.

In contrast to positive findings, Feller et al (40) failed to demonstrate that either vitamins A or C had any influence on the capacity of nasal secretions to inactivate influenza virus or affect the titer in serum of neutralizing antibodies for influenza virus, or affect the phagocytic activity of polymorphonuclear neutrophilic leucocytes for pneumococci.

Others have studied and reported (41, 42, 43) on the virucidal effect of ascorbic acid. It has been known for some time (44) that hydrogen peroxide is a product resulting from oxidation of ascorbic acid in air and Klein (43) believes that the virucidal effect of ascorbic acid is due to released hydrogen peroxide, not to any specific effect of the vitamin per se.

For a comprehensive review of the relation of ascorbic acid to infection the reader is referred to the review by Bicknell and Prescott (45). In general it appears that while routine administration of liberal quantities of ascorbic acid to persons suffering from pneumonia, diphtheria etc. is desirable, the evidence that the vitamin actually exerts a specific effect on infections is unsatisfactory. In that connection the American Medical Council on Pharmacy and Chemistry has ruled:

> "Unless more convincing evidence is presented than is now available, no claim referable to the anti-infective effect of ascorbic acid will be recognized."

Ascorbic Acid as a Detoxicant

Reference has already been made to the effect of ascorbic acid on the detoxifying power of the liver (See p. 304). There are other evidences of the value of ascorbic acid as a detoxicant.

In 1941 Bundesen et al (46) reported an action of ascorbic acid on reaction to neoarsphenamine. Sulzberger and Oser (47) had previously reported on the influence of ascorbic acid in the sensitization of guinea pigs and Dainow (48) recommended its use for this purpose in human subjects.

Bundesen et al (46) showed that solutions of arsphenamine or mapharsen could be completely protected from oxidation for 24–48 hours by addition of ascorbic acid and the significance of this observation became apparent as they began to make patch tests on patients hypersensitive to arsenical toxicity. Of a large number of patients treated, 38 out of 115 had a positive cutaneous reaction to a 30 per cent neo-arsphenamine solution. In 32 of these cases the cutaneous reaction was completely repressed by addition of ascorbic acid.

The number of patients who exhibit severe symptoms of intolerance to arsphenamine is considerable. Detection and prevention of such symptoms is of primary importance. The tests reported indicated that the reactions of hypersensitive patients can be fully prevented if the ascorbic acid content of the blood is maintained at a sufficiently high level. Another practical result of these studies was to show, hoever, that if a patient reacted to a patch test with 30 per cent arsphenamine containing 10 per cent ascorbic acid it would be hazardous to attempt sensitization with ascorbic acid.

In their original paper Sulzberger and Oser (47) suggested that ascorbic acid could stimulate specific antibody production. Additional evidence that ascorbic acid intake is of importance in determining complement titer has been provided by Ecker et al (49). They demonstrated that, while ascorbic is not identical with complement, it is an essential factor in immunity; that the optimum blood level is 1 mg. per 100 ml. of serum. The mechanism suggested is some relation to the reducing action of the vitamin.

Ascorbic acid has been reported as of value in the detoxification of certain industrial poisons. Benzene is a widely employed, potentially toxic, industrial solvent. Signs of benzene poisoning include the production of petechial hemorrhages similar to those observed in scurvy. It has also been shown that ascorbic acid can play a role in the breakdown of amino acids such as tyrosine and phenylalanine, both of which contain the benzene ring. In 1944 Eckman (50) suggested that ascorbic acid might exert a protective action against benzene poisoning and the problem was further studied by Forssman and Frykholm (51). The latter concluded that:

> "Exposure to benzene produces an increased requirement for vitamin C and an extra supply of vitamin C gives increased resistance to the effects of benzene vapors."

That ascorbic acid might have a beneficial effect in the treatment and prevention of lead poisoning was studied by Evans et al (52). But in this study they could find no clear evidence to justify recommendation of ascorbic acid as a means of minimizing lead absorption.

Pharmacodynamic Effects of Ascorbic Acid

Fliederbaum and Tislowitz (53) have reported that injection of the vitamin caused a diuresis associated with an increase in the blood colloid osmotic pressure. They attributed the action to: "the polyphasic effect of ascorbic acid on the colloid osmotic pressure of the blood." Shaffer (54) has also claimed diuretic action for ascorbic acid based on the observation that a combination of 500 mg. of the vitamin in addition to 5 ml. of mercuropurin produced from one half to two and one half times the diuretic effect of the mercuropurin alone.

Ascorbic Acid and Hay Fever

In 1942 Holmes (55) reported findings that gave promise of relief to hay fever victims through ascorbic acid dosage. The method consisted of daily doses of 200–500 mg. of ascorbic acid for a period of a week and then cessation until symptoms might again appear; in that event the treatment was repeated. The dosage, according to Holmes, protected not only against hay fever sensitivity but also against food allergies and asthma.

Some investigators have duplicated Holme's results, others found it impossible to do so. The matter needs much more study.

Ascorbic Acid and Reproduction

Phillips and coworkers (56) have produced evidence that ascorbic acid promotes the motility of spermatozoa. In experiments with bulls they were able to induce sperm motility by subcutaneous injections of ascorbic acid. The normal ascorbic acid content of bull plasma was 0.2–0.4 mg. per cent; of normal semen 3.0–8.0 mg. per cent. They showed that when the ratio of the ascorbic acid in the serum to that in the blood plasma fell below 40 the result was impotence.

Ascorbic Acid as an Anti-oxidant

A practical use of the reducing power of ascorbic acid has been found in its ability to prevent color changes and off flavors when incorporated in canned and frozen fruits. A sirup containing 0.2 percent of ascorbic acid is effective.

The reversible oxidation-reduction action of ascorbic acid naturally suggested that it might play a role in respiratory enzymes of some sort. King (57) has discussed this viewpoint in detail and stated:

> "A strong point against the theory that vitamin C functions as a major respiratory catalyst is the fact that depleted tissues do not show a decreased respiration capacity, and when ascorbate is added to the depleted tissues there is no rise in true oxygen consumption."

At any rate, there has been up to this writing no production of any enzyme complex containing ascorbic acid as the prosthetic group. Further study of the effect of ascorbic acid on tyrosine may develop such evidence.

Stigmata Indicating Ascorbic Acid Need

The Council of the American Medical Association has listed the following stigmata as suggesting ascorbic acid deficiency in man:

1. *Redness, edema, tenderness and bleeding on pressure of gums.*
   Observed in acute or subacute deficiency of moderate severity. Sometimes with, but usually without, other signs of scorbutic acid deficiency.
2. *Thickening and increased firmness of the gums.*
   With recession and exposure of the base of the teeth, including recession of interdental papillae; observed in chronic deficiency.
3. *Retraction of the gums.*
   Leaving pockets between gums and teeth; secondary infection resulting in pyorrhea. Observed in chronic deficiency.
4. *Loosening and shedding of teeth.*
5. *Increased capillary fragility.*
   Manifested by petechial hemorrhages of the skin, especially in the tourniquet test; observed in more severe and subacute deficiency. Easy

bruising, spontaneous ecchymosis of the skin, idopathic hemorrhage into joints and slow healing of wounds. Observed in severe, acute and subacute deficiency.

As the preceding pages indicate, ascorbic acid has been cited as beneficial in quite a range of diseased conditions, but most of these reports fail to provide satisfactory evidence of specificity of the vitamin in the effect reported. For that reason the Council on Pharmacy and Chemistry of the American Medical Association has published (58) the following statements regarding therapeutic claims:

> "Ascorbic acid is acceptable for the correction and prevention of scurvy. Definite claims for the therapeutic value of ascorbic acid should be permitted only in relation to scurvy until further chemical or experimental evidence has substantiated its usefulness in other state. . . .An optimum amount of ascorbic acid should be supplied at all ages for its therapeutic value in preventing the development of acute or latent scurvy. . . .Advertising of ascorbic acid for such symptoms as failure to gain weight or stoppage of growth, anorexia, anemia, infections, symptoms referable to the central nervous system or hemorrhagic conditions cannot be accepted unless it is definitely stated that the symptoms are referable to a demonstrated deficiency of ascorbic acid."

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SECTION II. FORMS AND CHEMISTRY OF ASCORBIC ACID

A primary requisite for studying the chemical and physiological nature of ascorbic acid was supplied by Holst and Fröhlich (1) who first produced experimental scurvy in the guinea pig. Their method was made more quantitative by diet modifications introduced by LaMer, Campbell and Sherman (2). This bioassay method permitted quantitative estimation of the ascorbic acid in a given source, but separation of the vitamin was made difficult by its extreme sensitivity to oxidation.

By 1931, however, considerable progress had been made, notably by Zilva (3) and King and associates (4), in the concentration of the vitamin. In fact, in 1932 Waugh and King (5) announced the isolation and identification of ascorbic acid from lemon juice. The isolated product had a protective level of 0.5 mg. per day. The product was called at the time "hexuronic acid". This same hexuronic acid was prepared from cabbage, oranges, and adrenal glands by Szent-Györgyi (6) in 1928 who is therefore credited with the first isolation of what was later to prove (7) to be protective against scurvy.

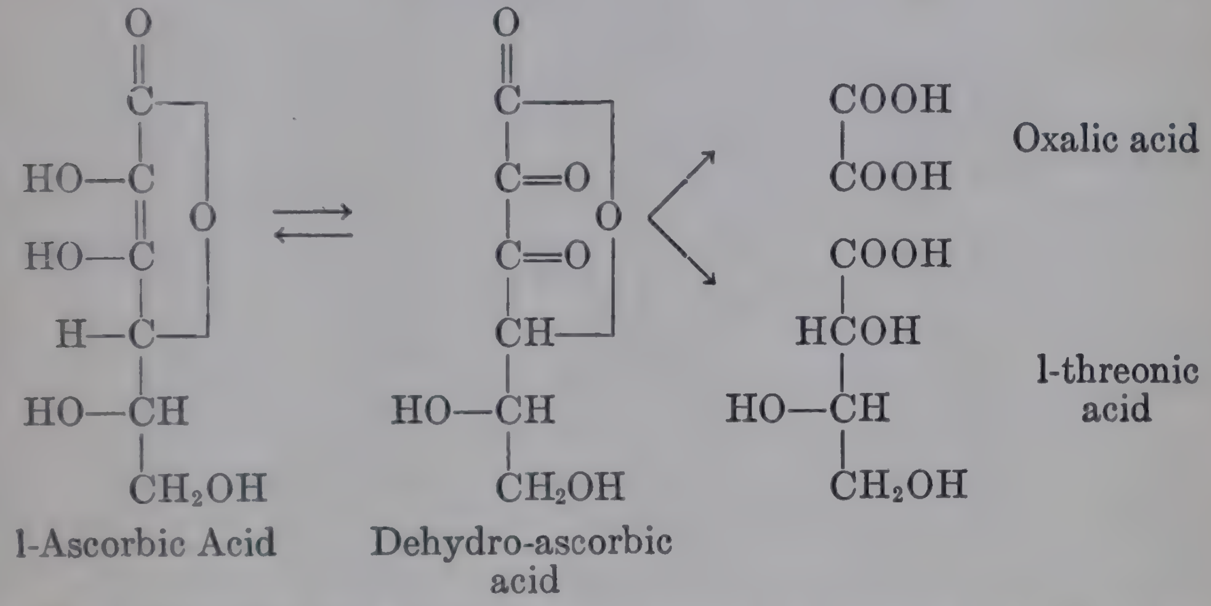

FIG. 33. STRUCTURE AND PROGRESSIVE OXIDATION PRODUCTS OF L-ASCORBIC ACID

The structural formula shown in figure 33 was established in 1933 (8, 9, 10, 11).

The material used was mainly from Szent-Györgyi's laboratory. But, even before the structure was fully confirmed Reichstein et al (12) succeeded in synthesizing the d- form and later the l- form starting with xylose (See figure 34). Later sorbose was used as the starting material.

In 1933 Szent-Györgyi and Haworth (13) suggested changing the name to ascorbic acid and that name has persisted, though for a time in this country it was known as "cevitamic acid".

Properties of ascorbic acid

The l-ascorbic acid form is the active vitamin C. As shown in figure 33, it is reversibly oxidizable to a form known as dehydroascorbic acid. Because this action is reversible and because sources of vitamin C may contain both these compounds it is necessary in assaying sources of vitamin C for potency to measure their content of both l-ascorbic and dehydroascorbic acid. If however oxidation proceeds farther the antiscorbutic potency is lost and the end products are oxalic acid and l-threonic acid (See figure 33).

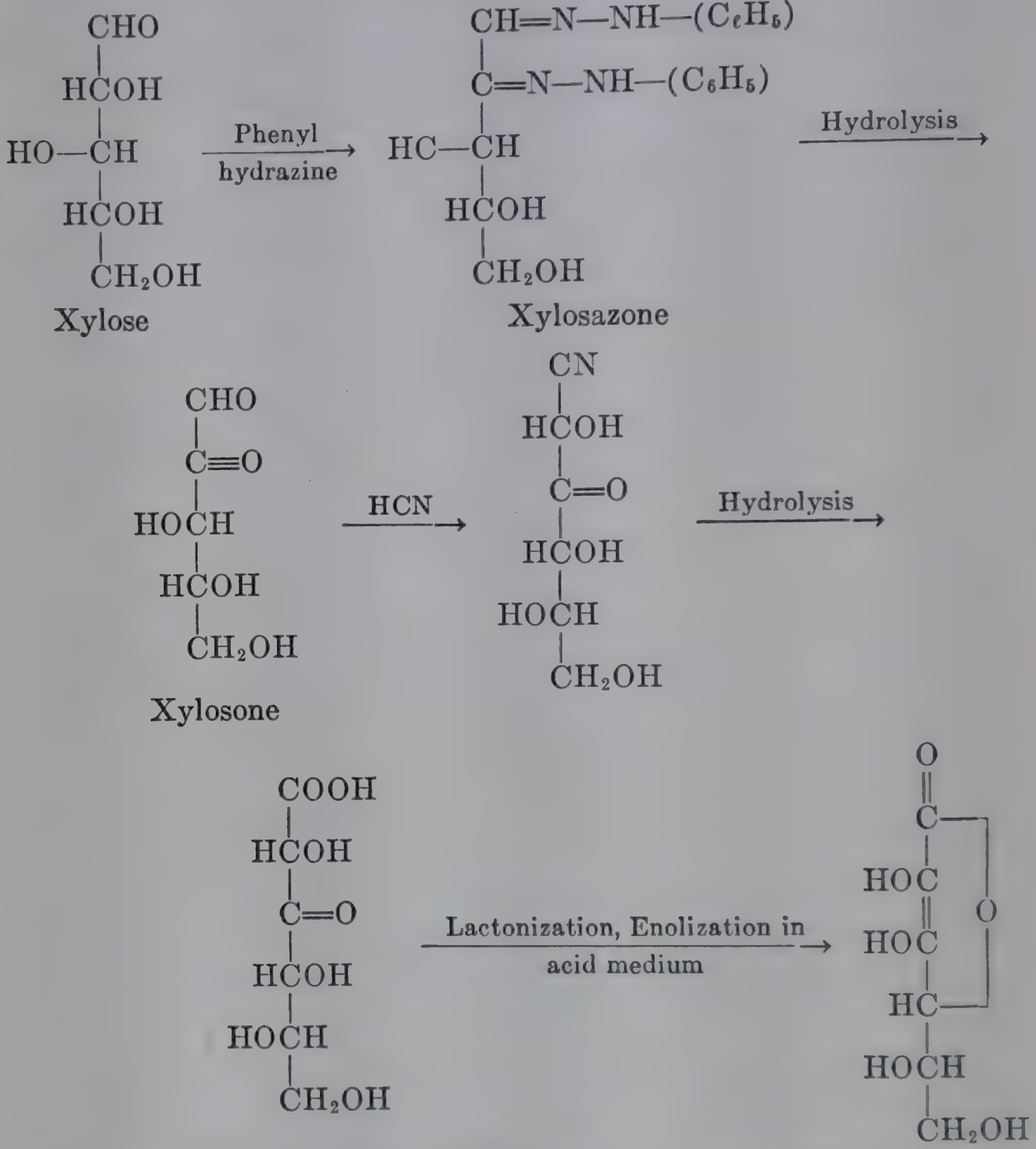

FIG. 34. A METHOD OF SYNTHESIS OF ASCORBIC ACID

The commercial l-ascorbic acid or vitamin C is about 99 per cent pure. It comes in the form of white odorless crystals or crystalline powder with a melting point of 189–192° C. It has an optical rotation of $\alpha[^{20}_{D}$ + 21–22° C. One gram dissolves in 3 cc. of water, 25 cc. of alcohol, 50 cc. of absolute alcohol and 100 cc. of glycerine. It is insoluble in benzene, chloroform, ether, petrol, benzin and fats. Potency is usually expressed in weight but occasionally in International units; 1 gram equals 20 000 I.U. It has maximum absorption in water solution at 260 $\mu\mu$ and at 263 $\mu\mu$ in alcohol solution.

As shown in figure 33 ascorbic acid is a sugar acid with a pH of 3–2 for concentrations increasing from 5–50 mg./cc.; molecular weight 176.06. Acidity appears to be due to dissociation of an enolic hydrogen rather than to the opening of the lactone ring.

In dry crystalline form ascorbic acid retains its potency quite satisfactorily, but in water solution it rapidly deteriorates in the presence of air, especially if exposed to light. Light destruction is also accelerated by the presence in the solution of flavins. Oxidative destruction is also accelerated by alkalies and the presence of certain metals, especially copper. Oxidation is retarded by acids, hydrogen sulfide and cyanides.

Dye Measurement of Ascorbic Acid Content

In 1930 and 32 three chemists in Frankfurt, Germany, made a contribution (14) that materially speeded up the isolation of the vitamin and also made possible a rapid assay of sources for ascorbic acid content. These three chemists, Tillmans and the Hirsches, had the problem of distinguishing between fresh and stale, natural and artificial fruit juices. For this purpose they used an oxidation-reduction indicator, a dye known as 2,6-dichloro-phenol-indophenol. Fresh and natural fruit juices gave a strong reaction with the indicator; stale and artificial juices little or no reaction. In 1930–32 they postulated that the substance in the natural fruits that produced this reaction was the antiscorbutic vitamin and that theory was confirmed. Today the dye titration method is used to measure food content of the vitamin and has made possible a wide knowledge of the vitamin's distribution in our dietary material. In figure 35 is given the chemistry of the reaction of the dye with ascorbic acid.

Other Forms of Ascorbic Acid

Ascorbic acid occurs in other than the active l-ascorbic acid form. In Table 54 are listed some of these forms and their relative antiscorbutic potency. It would appear that potency is associated with the position of the lactone bridge; in general the d- forms are inactive, the l- forms more or less active. But, that the ring position is not the only factor influencing antiscorbutic potency is shown in the table, for no l-form other than l-ascorbic acid has yet been found to have the full potency of the active vitamin.

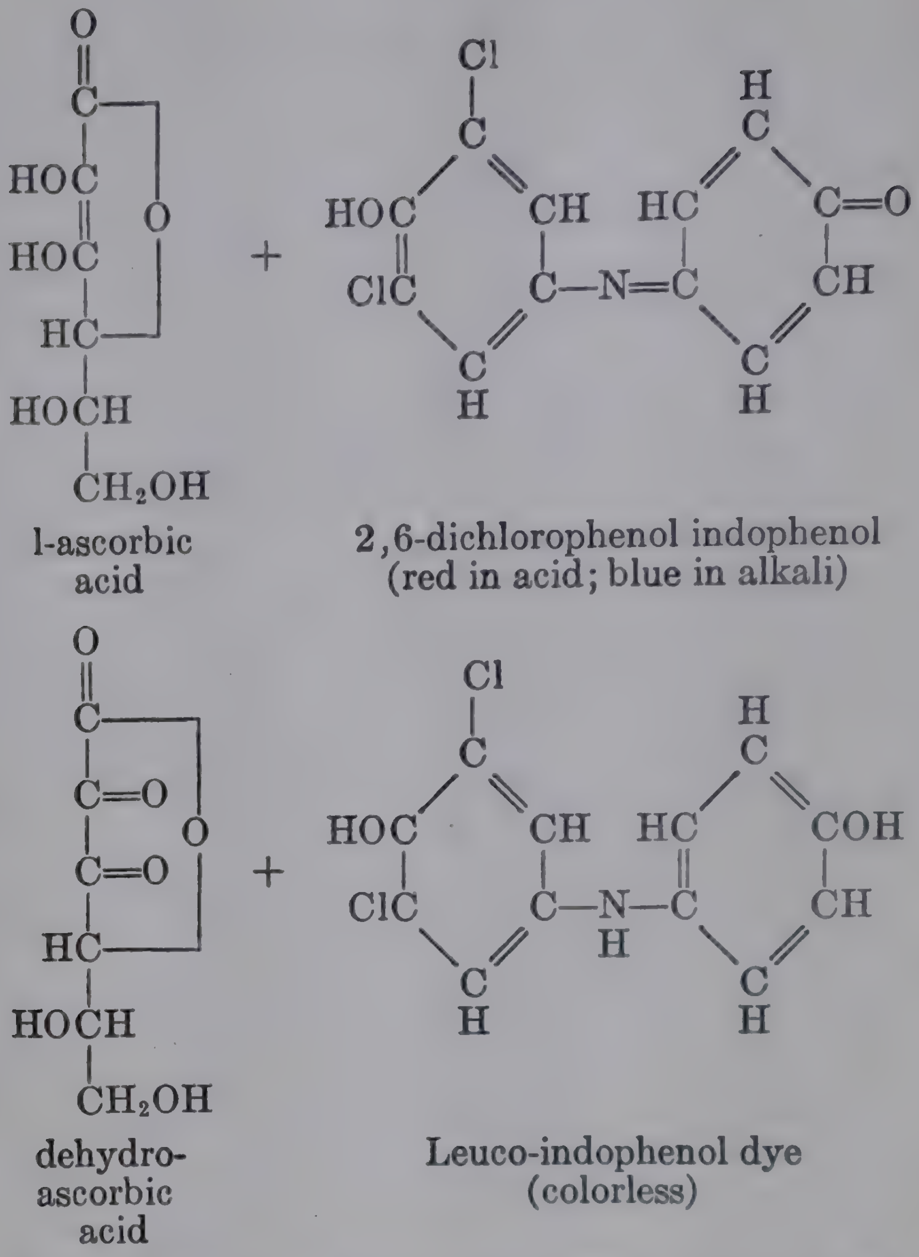
l-ascorbic acid
2,6-dichlorophenol indophenol (red in acid; blue in alkali)
dehydro-ascorbic acid
Leuco-indophenol dye (colorless)

FIG. 35. REACTION OF ASCORBIC ACID WITH INDICATOR DYE

TABLE 54

Forms and relative activity of ascorbic acids

Forms	Relative activity
l-ascorbic acid (vitamin C)	100
d-isoascorbic acid	5
d-ascorbic acid	None
d-glucoascorbic acid	None
d-galacto ascorbic acid	None
l-rhamno ascorbic acid	20
l-arabo ascorbic acid	5
l-gluco ascorbic acid	2.5
l-galacto ascorbic acid	1.0

Two of these forms, d-isoascorbic acid and l-glucoascorbic acid, have special interest.

While d-isoascorbic acid is very low in antiscorbutic potency (less than $\frac{1}{20}$th the potency of l-ascorbic acid according to Esselen et al (15)) it is more readily oxidized than l-ascorbic acid. For that reason it has been recommended for use in canned and frozen foods to both prevent browning and loss of the l-ascorbic acid content. However, Tressler and DuBois (16) report that they found l-ascorbic acid more effective than d-isoascorbic for protection against browning.

L-glucoascorbic acid actually blocks the action of the natural vitamin. It would therefore appear to belong in the category of vitamin antagonists. Woolley and Krampitz (17) have reported that feeding l-glucoascorbic acid to mice produced symptoms quite similar to those of scurvy. The first symptom was a severe diarrhea and change in color of the feces from black to golden brown. The anal region became red and swollen and at about the 7th day extensive subcutaneous hemorrhage appeared, at first on the chest and then on the legs, tail, and sides and in the gingiva; expecially below the lower incisors. On autopsy, lungs and joints of legs and ribs were fiery red the wrists and knees swollen and the legs edematous. Bones were poorly calcified and death ensued in all the animals, though the teeth did not loosen.

A peculiar effect was that on only 5 per cent glucoascorbic acid the animals eventually began to gain weight and signs of the disease actually regressed. This spontaneous cure ceased when the glucoascorbic dose was raised to 10 per cent and again developed. Omission of the glucoascorbic acid from the ration resulted in rapid cure.

That the action of glucoascorbic acid is specific seems indicated by the fact that no such effect followed the introduction of 5 per cent sodium-arabo ascorbic acid in the diet. There is no direct evidence that glucoascorbic acid actually competes with the natural vitamin as pyrithiamine competes with thiamine, and it is still not clear just how glucoascorbic acid blocks the function of the natural vitamin.

In 1945 Bannerjee et al (18) reported that 2 per cent of liver powder in the diet of either rats or guinea pigs receiving glucoascorbic acid would prevent the disease syndrome and they were unable to counteract its effect with l-ascorbic acid. They suggest that glucoascorbic acid does not exert its effect as an anti-vitamin C but by depriving the animal of some dietary factor that is present in liver.

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Section III. Evaluation of Ascorbic Acid in Human Nutrition

Classical scurvy is today of rare occurrence, but subclinical scurvy or mild vitamin C deficiency is still fairly prevalent due to too little consumption of fruits and green vegetables. Stress in dietetics has been put upon educating the people as to the importance of green salads and fruits and vegetables.

During World War II dietetic education was condensed into what was called the Basic Seven Plan of Food selection. (See Table 55). The person who conscientiously follows that plan runs little chance of vitamin deficiency and of vitamin C deficiency in particular. Groups I, II, and III are the foodstuffs which provide insurance against vitamin C deficiency.

Starting a little before World War II there was also an extensive development of dietary supplements in the form of capsules and tablets of vitamins. We know today that the preferred method of obtaining our vitamin and other dietary needs is by proper selection of natural foods but these tablets and capsules have provided insurance against faulty food selection and also material for medical prescription to correct specific deficiencies.

To protect the public in the selection of these products the Food and Drug Administration has set up standards and specific label statement requirements. The producer may state the content of vitamins in terms of weight but he must also state on the label what part of the minimum daily requirement of each vitamin is provided in each unit of product. The minimum requirements were published by the Food and Drug Administration and are shown in Table 55. It will be noted that these prescribe only 30 mg. daily of vitamin C as the minimum daily requirement of an adult. As study of needs progressed this allowance appeared too small and in 1942 the Food and Nutrition Board of the National Research Council for the first time published the amounts of vitamins and other nutrients considered desirable for people of all ages and sexes. These are suggested allowances to insure safety, *not requirements!* Their recommendations in regard to the various vitamins and other nutrients have been modified twice: the first set was given in 1942, revised in 1945 and again in 1948, but as will be seen in Table 56 the allowances for vitamin C have remained the same. How were these allowances established?

TABLE 55

I. The basic seven plan. Serve daily at least one item from each of the following groups:

Group I. Green and yellow vegetables. One raw, one cooked, frozen or canned.
Group II. Oranges, tomatoes, grapefruit, or raw cabbage or salad greens.
Group III. Potatoes and other vegetables and fruits. Raw, dried, cooked, frozen or canned.
Group IV. Milk and milk products. Fluid, evaporated, dried milk and cheese.
Group V. Meat, poultry, fish or eggs. OR dried peas, beans, nuts, or peanut butter.
Group VI. Bread, flour and cereals. Natural whole grain, enriched, or restored.
Group VII. Butter or vitamin A fortified margarine.

II. U. S. Food and Drug Administration's Standards for Label Control.

Vitamins	Minimum amounts required for an adult per day
A	4000 U.S.P. units
B_1 (thiamine)	1.0 milligrams
B_2 (riboflavin)	2.0 milligrams
C (ascorbic acid)	30 milligrams

Bases for Estimating Vitamin C Allowances

Three criteria have been used in estimating vitamin C allowances;

1. Urinary excretion data; basal and after test dose.
2. Blood content of ascorbic acid.
3. Hemorrhagic diathesis or capillary fragility tests.

The nature of the problem of estimating human needs and the methods available were reviewed by Sybil Smith in 1938 (1). In that review she noted that the first attempts at using human subjects in the measurement of ascorbic acid requirements were made by Göthlin (2) in Sweden in 1931. He used a measurement of capillary fragility and this method was later modified by Dalldorf (3). In 1933 Harris, Ray and Ward (4) initiated procedures based on measurement of urinary excretions of the vitamin and i 1936 Farmer and Abt (5) published a method of measuring the blood leve of the vitamin.

Urinary Saturation Tests

In 1935 Abbasay et al (6) used the term "resting level" to indicate day-by-day urinary excretion of ascorbic acid. At that time they set 10 mg. as the border line between deficiency and adequacy; 20 mg. to indicate a moderately low intake and 40 mg. as indicating a liberal intake.

TABLE 56

Suggested daily allowances of ascorbic acid

(Food and Nutrition Board of the National Research Council)

Individuals	Daily ascorbic acid allowances		
	1942	1945	1948
	mg.	*mg.*	*mg.*
Men	75	75	75
Women	70	70	70
Pregnant women	100	100	100
Lactating women	150	150	150
Girls 13–15 yrs.	80	80	80
Girls 16–20 yrs.	80	80	80
Boys 13–15 yrs.	90	90	90
Boys 16–20 yrs.	100	100	100
Children up to 12 yrs.			
Under 1 year	30	30	30
1–3 yrs.	35	35	35
4–6 yrs.	50	50	50
7–9 yrs.	60	60	60
10–12 yrs.	75	75	75

A little later they concluded that if a subject excreted less than 13 mg. per day and failed to respond by marked increase to a test dose of 700 mg. per 140 lbs. of body weight the diet contained less than the minimal requirement of ascorbic acid. On this basis 13 mg. urinary daily excretion was set as indicating just protective intake. But, van Eekelen raised this figure to 40 mg. per day to indicate adequate tissue saturation (7).

The socalled saturation test has become today one of the principal means of determining adequacy of vitamin C intake. In practice it was found that if 50 per cent of a large test dose appeared in the urine within 24 hours after administration the subject might be considered to have been taking an adequate amount of the vitamin in his daily diet. Failure to excrete that percentage of the test dose indicated need for increase in daily intake.

A typical test of this sort was reported by Roberts et al (8) in 1943. They used a test dose of 300 mg. of ascorbic acid. Their test subjects were girls 6–13 years of age and as stated above the criterion of saturation was recovery of 50 per cent of the test dose in the urine within 24 hours after administration. On the bases of these tests they reported that these children required an intake of at least 62 mg. of ascorbic acid daily to produce saturation. However there were wide variations in individual responses to the test.

Other investigators have reported similar variations. Belser et al (9) with tests on 7 adults reported the saturation requirement as 1.0–1.6 mg. per kilo of body weight; Todhunter et al (10) 1.6–1.7 mg. per kilo of body weight in 3 adults; Storvick and Hauck (11) 1.33, 1.32, 1.34, 2.85, and 1.51 mg. per kilo in 5 subjects respectively; Lewis et al (12) 1.35, 1.26 and 1.22 mg. per kilo in 3 adults.

TABLE 57

Ascorbic acid saturation requirements

(After Kline and Eheart (13) 1944)

Subjects	Weight	Age	Saturation required per kilo body weight	Daily intake necessary for saturation
	kilos	*years*	*mg.*	*mg.*
1	49.8	31	2.2	109.5
2	46.8	21	1.4–1.8	65–84
3	70.0	18	1.4–1.8	98–126
4	55.0	35	1.4–1.8	77–29
5	46.0	21	1.4–1.8	67–87
6	64.8	21	2.2	Over 142.5
7	49.3	20	1.4–1.8	54–88
8	54.1	22	0.6	32
9	63.6	20	1.4–1.8	89–114

Kline and Eheart (13) have reported a study of 18 subjects tested by the criterion of 50 per cent recovery of test dose in 24 hour urine excretion as follows: 17 required between 1 and 1.7 mg. per kilo of body weight but one had to take at least 2.85 mg./kilo to meet the test. For the 17 men this would mean a daily intake of from 70–138 mg. per day. They then modified their test as follows: They selected 9 normal young women ranging in weight from 49.2 to 70 kilos. Two were 31 and 35 years old respectively; the rest 18 to 22. Their diets during the test period supplied from 15.43 to 16.18 mg. of ascorbic acid per day. Urine collections were made only during the 24 hours preceding and following the test dose. The test period was a five day saturation period and during this period 200 mg. of ascorbic acid was given for 4 days and 500 mg. on the fifth day. If 50 per cent recovery in the urine resulted on the next day the subjects were considered saturated. Then the subjects were started on an experimental 6 day period on the controlled diet plus various ascorbic acid supplements. By this method 7 subjects were saturated at 1.4–1.8 mg./kilo; one by 0.6 mg./kilo; and two required more than 2.2 mg./kilo. These results adjusted to weights are shown in Table 57. With one exception the upper limits are distinctly above the N. R.C. optimal allowances and it is evident that weight and age are not the sole determinants of vitamin C requirement.

Blood Level Measurements

Examination of blood content led Abt and Farmer (14) to postulate that a content lower than 0.5 mg. per 100 ml. of blood indicated deficiency, and that a content of 1.0 mg./100 ml. probably indicated adequacy of intake. Studies have been made using these criteria but as in the case of the urine saturation tests there is variation in individual response. Roberts et al (8) reported on 30 girls 6–12 years and found 62–72 mg. intake per day maintained a blood level of 0.67 mg./100 ml. Bessey and White (15) tested 93 children and found that they maintained a blood level of 1 mg./100 ml. on 45–50 mg. intake per day; 30 mg. to maintain at least 0.6 mg./100 ml. Kyhos et al (16) tested 71 subjects and found that 75 mg. per day intake maintained a blood level of 1.0 mg./100 ml. and that 50 mg. intake produced 0.6 mg./100 ml. But they also called attention to the fact that while plasma values are the first to fall when deficiency state develops they are slow to return to normal after the intake is increased. Fincke and Landquist (17) set 0.8 mg./100 ml. as indicating normality and found an intake of 0.8–1.2 mg./kilo necessary for its maintenance. That would mean 56–84 mg. per day for a man weighing 40 kilos. Dodds and McLeod (18) studied two groups of college women, 196 in one group and 149 in another group. The relation between intake and blood levels differed in the two groups. In the 196 group the intake was 82.9 ± 2.65 mg. per day and the blood levels averaged 0.64 mg./100 ml. In the 149 group the intake was 86.3 mg. ± 3.14 daily and the blood levels averaged 0.84 mg./100 ml. There was also wide variation within the groups.

From these and other observations it is probably safe to assume that if an individual shows a blood level below 0.6 mg./100 ml. he needs a greater intake. But in using the blood level to estimate saturation, blood levels should be taken over a considerable period of time to get a true picture of the effect of intake increases or adequacy.

The Capillary Fragility Tests

As will be discussed in more detail in Chapter XVIII, ascorbic acid deficiency is not the only factor involved in production of capillary fragility. Hess (19) first showed that if one applied a tourniquet and thus increased capillary pressure, bleeding with production of petechial hemorrhages resulted in scorbutic individuals. Göthlin, as already stated, was the first to make use of this technique to diagnose scorbutic condition, but Dalldorf (3) worked out an instrument for this purpose which measured response to reduced pressure. He set an average of 35 cm. of mercury of pressure without appearance of petechiae as indicating normal intake of ascorbic acid; 25 cm. as indicating inadequate intake. This method, like the blood tests, gives results of value for diagnosis of deficiency but lacks quantitative accuracy when used to determine saturation. Of it Dalldorf himself has said:

> "The virtue of the capillary fragility test is that it is a measure of scurvy and capillary fragility due to vitamin C depletion. As identified by a test dose of vitamin C followed by observations of the resistance it is prima facie evidence of a pathological degree of depletion. This the chemical tests give only by inference. There is no reason to believe that it is precise or uniform to any greater degree than other measurements of body function and much of the criticism of it has been from individuals who have looked for a degree of precision that the test lacks."

The Rotter Intradermal Test

Another means of diagnosing ascorbic acid deficiency was proposed by Rotter (21) in 1938. In his original procedure the test consisted of injecting subcutaneously a solution of 2,6 dichlor-phenol-indophenol and noting the time required for it to be decolorized. He considered that if the decolorization occurred in 5 seconds, the subject was not deficient in ascorbic acid. If it took ten seconds or more, deficiency was indicated. This test has not gained general acceptance. Poncher and Stubenrauch (22) studied its use with the following conclusion:

> "The intradermal test in its present form cannot be relied upon to give satisfactory clinical information as to ascorbic acid saturation in the individual case."

Slobody, (23) however, reported a modification of the test that he claims does parallel the degree of body saturation. He showed that raising a wheal of 4 mm. with 300th normal dye solution indicated a subnutrition state. Of 59 patients with blood levels below 0.3 m./100 ml. the skin test time was more than 14 minutes. More than 4 minutes he considers to indicate deficiency.

Portnoy and Wilkinson (24) have also reported on use of the intradermal test but interpreted the results as follows: Discolorization in less than 5 minutes to indicate saturation; in 5–10 minutes normality; more than ten minutes definite deficiency.

Perhaps the most careful test of the Rotter procedure has been by Holland et al (25). They made a detailed comparison of results by the intradermal test with plasma ascorbic acid content and urinary excretion tests on 34 hospital patients and 14 normal individuals. They report serious technical difficulties in making the test. Also a high degree of discrepancy appeared when compared with saturation tests. The technical difficulties included allergic reactions, variations when the test was made simultaneously on both arms of a subject, and difficulty in setting the end point of the discolorization. Only between the fasting plasma level and the 5 hour urinary excretion of ascorbic acid did they get satisfactory correlation.

Basis of the N.R.C. Allowances

While it is admitted that individuals vary in response to saturation tests and blood tests, these have been the main reliance of the Food and Nutrition Board in formulating the allowances shown in Table 56. Jeans (26), for example, in commenting on these allowances has stated:

> "Knowledge of the ascorbic acid requirement is in a more satisfactory state than that for any other vitamin, even so, a few problems still exist, such as decision as to the appropriate blood level or as to the amount excreted in the urine as a criterion of satisfactory stores."

Dalldorf (20) has also the following comment to make on the Board's allowances:

> "In explaining the problems faced by the Committee on Food and Nutrition of the National Research Council, Roberts points out that while the amount needed to maintain blood levels is known as well as the greater amount needed for saturation, it is not known what a normal blood level is or whether saturation is necessary or even desirable. The Committee took the compromise position and recommended 75 mg. for adults, 100 mg. during pregnancy, 150 during lactation and a scale of 30 mg. for infants under one year to 80 mg. for adolescent children.
>
> The problem is a little different from that of the requirement of other vitamins. In the case of ascorbic acid, however, there is clear proof that in experimental animals an intake twice or three times that required to prevent clinical symptoms is necessary to prevent anatomical stigma in certain organs and from general biological principles the concentration of vitamin C in human milk, the concentration in certain organs and the ready availability of large amounts in foods, it would seem that the Committee's recommendations are reasonable and justified.
>
> In individuals the problem is different. It is worth while noting that irregularities in excretion, blood levels, and all other measurements emphasize, in the case of vitamin C, the wide range of individual variations. They have been recognized by clinicians for years."

Availability of Ascorbic Acid

As previously stated, thanks to the dye titration test, the distribution of ascorbic acid in natural foods is quite well established and available in published tables. It is known, however, that vegetable sources contain an ascorbic acid oxidase capable of inactivating ascorbic acid. In the case of such foods being eaten, does the oxidase operate after ingestion?

Hochberg et al (28) have reported that, despite the fact that in vitro ascorbic acid oxidase rapidly destroys large quantities of ascorbic acid, no great destruction of either naturally-occurring, dietary ascorbic acid or of extra ascorbic acid was noted in vivo due to this factor. Melnick and Oser (29) state that:

> "Apparently ascorbic acid oxidase in the fresh fruit and vegetables consumed is destroyed or its activity inhibited in the gastro-intestinal tract. It has been demonstrated that ascorbic acid oxidase can rapidly oxidize ascorbic acid in vegetables to and beyond the dehydroascorbic stage. The catalytic effect of copper upon the oxidative destruction of ascorbic acid is well recognized. However, human availability studies (30) have indicated that, in the digestive tract ascorbic acid and copper are not incompatible. No greater destruction of ascorbic acid occurred in vivo as the result of administration of extra-dietary copper."

It would therefore appear that in spite of presence of ascorbic acid oxidase in fresh fruits and vegetables we need not fear its effect on the fruit and vegetable sources of the vitamin we select. There is, however, much greater danger of loss of values through cultivation, transport, storage, cooking and method of serving. Examples of the seriousness of such losses are illustrated in Table 58.

It may be recalled that in the discussion of the relation of ascorbic acid to scurvy, Elmby and Warburg (31) reported a case that failed to respond to pure ascorbic acid but did respond to lemon juice. These investigators have postulated that natural foods furnish factors which promote the utilization of ascorbic acid. There has been considerable confirmatory evidence (32, 33, 34) of this viewpoint. However, the problem needs further study. Available evidence still further supports the contention that it is better to get our vitamins from natural foods.

Ascorbic Acid Requirements of Domestic Animals

The only animals which appear unable to synthesize their needs of ascorbic acid are man, monkey, and guinea pig. In the case of cattle the only occasion when supplementary ascorbic acid appears necessary is to promote sperm motility in bulls and prevent impotence. In these animals a definite blood level appears important and may require ration supplementing. (See Section I, p. 308).

In the case of chicks, Briggs et al (35) have reported that addition of ascorbic acid to a highly purified diet appeared to be growth promoting, while addition to the farm ration produced no such effect. They attribute the effect to the action of the ascorbic as a growth stimulant rather than as a growth factor.

Animals do require ascorbic acid, however, and must maintain a given blood level for health. Todhunter and McMillan (36) have given the following plasma content as indicating adequate synthesis and intake; 0.25–0.66 mg./100 ml. for healthy monkeys, guinea pigs, rabbits, goats, horses, sheep, dairy cattle, and dogs on adequate diets. In rats they found a higher level in the males than in the females which, as in the case of bulls and stallions, may have significance in the control of breeding capacity.

TABLE 58

Examples of factors that affect the availability of ascorbic acid in dietary foods

I. The place where they are grown:
  Hollandia Spinach grown on upland soil had 0.75 mg./gm.
  Hollandia Spinach grown on muck soil had only 0.42 mg./gm.

II. Temperature and time of storage affects it.
  Hollandia spinach stored at room temperature (73–79) lost 96% in 7 days.
  Hollandia Spinach stored at 34–37°F. lost only 38% in 7 days.

III. There are losses by heat destruction and leaching in cookery.
  Boiled cabbage was shown to lose 12 per cent loss by heat treatment; 66 per cent loss into the cooking water; and only 22 per cent retention in the cooked drained cabbage.

IV. There are losses due to delays in serving.

Foods	Per cent loss of ascorbic in 3 hours on the serving table
Cabbage	91
Spinach	96.4
Peas	95.7
String beans	76.0
Carrots	91.4
Squash	95.0

How is Ascorbic Acid Synthesized?

Smythe and King (37) in 1942 summarized the literature giving claims for demonstration of the synthesis of ascorbic acid in isolated tissues. The report of Roy (38) gives evidence that ascorbic acid synthesis is dependent on the products and at least on the process of glucose metabolism. But, if the synthesis depends on the recognized steps of the intermediary carbohydrate metabolism, there must still be some unexplained essential difference in those species which are unable to synthesize the vitamin.

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CHAPTER XVIII. VITAMINS P

Section I: Functions of Vitamins P

Szent-Györgyi's postulation of a vitamin P is perhaps best told in his own words: (1)

> "In citrus fruits we found a specially active flavonol present as a glucoside which up to that time had been unknown in this form. We called it with V. Brückner 'eriodictin'. In the unripe plant we found a substance in a methylated, inactive, stable form which had been known for a long time as hesperidin. . . .I had a letter from an Austrian colleague who was suffering from a severe hemorrhagic diathesis (vascular type). He wanted to try ascorbic acid in his condition. Possessing at the time no sufficient quantities of crystalline ascorbic acid I sent him a perparation of paprika (vitaprik) that contained much ascorbic acid and the man was cured by it.
>
> Later, with my friend St. Rusznyak, we tried to produce the same therapeutic effect in similar conditions with pure ascorbic acid but we obtained no response. It was evident that the action of the paprika was due to some other substance present in this plant. It would have been a hopeless job to try to isolate this substance had we not had our experience with flavones. So we set out to prepare flavones, in the first place eriodictin, that can be easily injected and we found that similar pathological conditions, not previously amenable to therapy, could be cured by it with regularity. The effect had several characteristics of vitamin action, so tentatively, I called it vitamin P in honor of paprika and permeability, on which later it was found to have an influence."

Citrin

Szent-Györgyi's first concentrate of vitamin P was made from lemons and to it he gave the name 'citrin', some times spelled citrine. This is not to be confused with the American pharmaceutical product of the same name, derived from watermelon seeds and originally called 'cucurbocitrin'. He believed citrin to be composed of two flavanone glucosides, namely hesperidin and another called eriodictin, and to the latter he ascribed the chief vitamin P activity.

Later Robiezniek (2) and Armentano (3) suggested that citrin contained a third flavone glucoside, the glucoside of quercitin. To this latter Armentano attributed the ability of citrin to lower blood pressure.

The glucoside eriodictin has not been isolated at this writing although its aglucone has long been known as a constituent of the plant Eriodictum Californicum, sive glutiosum (the Yerba Santa of the Mexicans). What part of the glucoside is responsible for prevention of capillary fragility is still unsettled, but several theories have been advanced.

In 1942 Wawra and Webb (4) reported the isolation from lemon peel of a new oxidation-reduction enzyme which they claimed to be the chalcone of hesperidin, and suggested that the vitamin P activity of citrin was due to this chalcone, not to eriodictin.

In 1943 Higby (5) methylated the chalcone form of hesperidin to such an extent as to result in a new drug of marked solubility, stability, and counteraction of capillary permeability.

Rutin

In 1920 Sando and Bartlett (6) described a yellow pigment obtained from the California poppy which they identified as 'rutin' or 'rutiside'; first described by Weiss in 1842. It was a glucoside of quercitin. Lloyd isolated a similar glucoside pigment from the white flowers of the elder which he called 'eldrin' but in 1924 Sando and Lloyd (7) proved rutin and eldrin chemically identical. On hydrolysis they yielded the aglucone quercitin, glucose and rhamnose. Along with other similar compounds rutin had been investigated by Lavollay and coworkers but more recently it has been especially investigated by workers in the Eastern Regional Laboratory of the U. S. Department of Agriculture (8). In confirmation of earlier work it was found, like citrin, to decrease capillary fragility under certain conditions. To date buckwheat has proved the most economical source of rutin, proving about 6 per cent in the leaves and blossoms, little in the stem, none in the seeds or buckwheat meal.

Other Flavone Derivatives with Vitamin P Activity

If one examines the molecular structure of a flavone glucoside (See fig. 36, Section II) it is shown to be a polyhydroxy phenyl chromone, alpha phenyl benzo-gamma-pyrone. The pyrone group in turn shows a relation to the catechins and anthocyans. In 1936 Szent-Györgyi et al (9) produced evidence that certain phenyl-benzo-gamma-pyrone pigments evinced vitamin P characteristics, while Sevin (10) reported the comparative vitamin P characteristics of a series of polyhydroxy derivatives of flavones.

Following this phase of the search for the character of the vitamin, Lavollay, Parrot and coworkers (11) have reported that epicatechin is more potent in vitamin P activity than citrin or any other substance. In 1943 Lavollay, Parrot and Sevestre (12) reported that the vitamin P activity of a crude catechin concentrate was due to traces of epicatechin. If d-catechin was epimerized (Freudenberg method) still greater vitamin P activity was acquired. Stability was increased and from the solution was obtained a mixture of crystalline isomers, mainly epicatechin with vitamin P activity in minute doses.

Still later (1944) Parrot, Lavollay, Sevestre and Galmiche (11) reported the separation (by fractional distillation) from crude catechin of d-epicatechin, for which they claim a vitamin P activity 500–1,000 times that of Szent-Györgyi's citrin.

In 1945 Lavollay (13) also reported that esculin (a glucoside from the horse chestnut) had vitamin P activity, and in that paper he suggested that the vitamins P be divided into two groups: P_2 for natural products derived from benzo-pyrone, and P_1 for similarly acting compounds of a phenyl-chroman structure.

To date the number of different substances with an effect on capillary fragility or permeability has become considerably increased. Javillier and Lavollay (14) list, in addition to citrin and rutin, quercetol, cyanidol, d-epicatechol, phloretol, coumarin, and esculetol as having the critical faculty and suggest that it is doubtful whether the term vitamin P should be retained.

Relation of Vitamins P to Vitamin C

As discussed in Chapter XVII ascorbic acid was shown to control certain types of hemorrhagic diathesis. To qualify as a vitamin P it was necessary to show ability to control a hemorrhagic diathesis that was not accomplished by ascorbic acid.

In 1943 Bourne (15) devised the following procedure: 54 guinea pigs were placed on a scorbutogenic diet. Then to each animal was given a protective dose of 10 mg. of ascorbic acid daily by mouth. At the start of the experiment 30 of the animals did not develop a hematoma at 460–560 mm. pressure*.

If with a given animal the chosen pressure level failed to produce the hematoma, another section of the skin was tested until the desired effect was obtained.

After three to eight weeks on the diet all the animals showed at least a 50 per cent decrease in capillary resistance, and in many cases an even greater decrease occurred. In 15 of the animals a daily intake of 10 mg. of citrin produced a rise in capillary resistance of 60–100 per cent in seven days. In the control animals the capillary resistance remained low. Although in a few instances there was a spontaneous rise in resistance, it was never over 20 per cent. Also, when citrin treatment was discontinued the capillary resistance reversed.

Scarborough et al (16) also reported human cases where capillary resistance was not attained by various vitamins, including C until hesperidin or citrin was administered. In one study 5 scorbutic patients were kept on a vitamin-deficient basal diet and fed various vitamin supplements. Administration of ascorbic acid cured gingival bleeding as well as subcutaneous and muscular hemorrhages, an effect not accomplished by vitamin P preparations. In spite of this effect of ascorbic acid, capillary resistance remained low and was brought to normal only after administration of citrin to which there was prompt response.

---

* Bourne used a 20 mm suction cup placed on the shaved vaselined skin. The pressure was lowered to a given level and released after ten seconds. The measure of capillary resistance was taken as the negative pressure at which sufficient petechiae developed so that a uniform reddish-purple hematoma occurred.

As early as 1941 St. Rusznyak and Benko (17) reported that rats (which do not require ascorbic acid) developed, when put on a scorbutogenic diet, an increased capillary fragility which was corrected by citrin.

Capillary Structure

Chambers and Zweifach (18) have discussed in detail the structure of the capillary and what must happen to it to vary permeability. They picture the capillary as a tube formed of pavement-like endothelial cells held together by an intercellular cement. This tube is lined by a layer of adsorbed plasma protein which may penetrate into the pores of the intercellular cement and thus modify their size. The tube is covered on the outside by a sheath that serves as a supporting layer and has characteristics common to the surrounding connective tissue matrix.

In the summary of their paper they suggest the following factors that may control permeability and fragility:

1. The inter-endothelial cement serves as a basic framework. It is more or less porous and the pores can vary in size with the electrolytic balance of the medium and may be affected by the chemical constituents of the blood. It would act as an ultra-filter and pore size would regulate the charater of the fluid that permeated it. The extent of the filtration would in turn be affected by the blood pressure and actual bleeding would require modification of the pore sizes to permit passage of both plasma and corpuscles.

2. The endocapillary lining appears to be an adsorbed layer of the blood proteins which by penetrating into the intercellular cement could modify the pore sizes.

3. The precapillary sheath is a condensation of connective tissue serving to give mechanical support and is probably sufficienctly porous to allow free passage of the fluid part of the blood.

Each of these structures can be affected separately by a variety of conditions which induce changes in the permeability of the capillary wall. Hence the authors say: "It is difficult to assume a single permeability factor, such as permeability hormone or vitamin".

Incidentally, the terms *capillary fragility* and *capillary permeability* have been used in the literature more or less interchangeably to characterize conditions that result in capillary bleeding. However, capillary permeability can occur without capillary fracture and it is generally held today that the term capillary fragility should be used only when there are lesions in the intercellular cement.

Theories on Action of Vitamins P

Clark (19) has reviewed the various theories concerning how vitamins P act, and the objections to these claims. Special attention has been given to the viewpoint of the French investigators who have postulated (14, 20, 21, 22) that vitamin P substances act as antioxidants and retard or inhibit the oxidation of adrenalin and adrenochrome, and thus maintain the vaso-constrictor action of these hormones. Adrenalin is recognized as the hormone that regulates the tone of the capillaries and if any exogenous substance is capable of modifying the resistance and permeability of the capillaries it would be reasonable to suppose that it might do so by action on adrenalin, modifying or continuing its vaso-constrictor effect.

It is also known that adrenalin is readily oxidizable and is oxidized with great rapidity in vivo with or without the action of enzymes. Bacq (23) showed in 1936 that the action of adrenalin could be brought to an early end by oxidation in the tissues. The evidence is the prolongation of its effect through injection of substances which in vitro were found to protect it from oxidation, viz. pyrogallol, catechol, hydroxyhydroquinone and thyroxine.

Clark (19) translates the French hypothesis as follows:

> "That the hormone for normal capillary tone, fragility, and permeability is adrenaline or more probably its unstable oxidation product adrenochrome. Adrenergic fibers, when stimulated, have been thought by Bacq, Roskam, and Derouaux, Sollman, Parrot and others, to transform adrenochrome into sympathin E. 'Vitamin P' is postulated to inhibit the in vivo oxidative destruction of adrenochrome and sympathin but much work remains to confirm this hypothesis."

Evidence Pro and Con the Adrenalin Antioxidation Theory

In support of the adrenalin or sympathin antioxidation theory, the French workers have reported Warburg manometric studies of adrenalin auto-oxidation, pharmacalogical studies of the enhancement and prolongation of adrenalin effects on excised mammalian smooth muscle, and in vivo experiments on effects of adrenalin on blood pressure, contraction of the nictitating membrane, and on nerve chronaxie. They have reported adrenalin and ascorbic acid antioxidative effect on the part of various substances which also decreased capillary fragility and permeability.

Clark (19), however, states that in his laboratory, in collaboration with Geissman, several dozen of pure and synthetic compounds were tested for their ability to prolong the effects of adrenalin upon excised mammalian smooth muscle and to inhibit in vitro formation of adrenochrome from adrenalin. Clark also found that many of the compounds, cited by the French workers to have marked effect on capillary fragility, were feebly active or actually inert in their tests: for example, phloretin, phloridizin and esculin. While the French workers reported d-epicatechin highly active and d-catechin and l-epicatechin inactive in preventing capillary fragility, Clark and Geissman's tests showed no significant differences in the adrenalin anti-oxidation effects of these compounds.

Clark and Geissman also reported that the antioxidant effect required that the compound contain the free ortho-dihydroxy groupings in the phenyl ring. If their view in regard to the essentiality of this structure is valid, the observations of Hughes and Parkes (24) raise further objections to the French theory. These investigators examined the several natural and synthetic vitamin P-like compounds for their ability to elevate the lowered capillary resistance of guinea pigs on a P-deficient diet containing adequate ascorbic acid. Of 13 synthetic chalcones only one contained the ortho-didydroxy phenolic structure necessary for in vitro adrenalin antioxidant power, yet many of their compounds had marked capillary resistance effect and one active compound (4-amino chalcone glucoside) had no free hydroxyls at all.

Sokoloff and Redd (25) have also challenged the French explanation of vitamin P activity, and state that in their experience the view that vasoconstrictive action is characteristic of vitamin P compounds has little if any physiological foundation. They also question the evidence that adrenalin increases capillary resistance. They say:

> "The only evidence we have been able to find in the articles of these contestants is the statement in their paper (14) entitled 'Les substances agissant sur la resistance et la permeabilité Capillaires et la notion de vitamin P' in relation to the following observation: 'One milligram of adrenaline increases vascular resistance in man from 30 cm normal resistance to 50–60 cm. of mercury at least for an hour. This action of adrenaline is durable. Adrenochrome posesses the same property'.
>
> Their observation does not prove any change in capillary resistance; it only proves that adrenaline possesses a vaso-constrictive action.
>
> The leading idea in Lavollay's work is that vitamin P factors delay oxidation of adrenaline and that there their usefulness as far as capillary permeability is concerned is ended. In other words, vitamin P factors have no direct effect upon either the capillary walls or capillary permeability. . . .Not only have they not proved that adrenaline maintains capillary tone; not only were they unable to demonstrate that adrenaline increases capillary resistance or decreases capillary permeability, but they have offered insufficient evidence in defense of their basic viewpoint about the delaying action of vitamin P by oxidation of adrenaline.
>
> It is true that Parrot and Cotereau have proved that vitamin P factors delay in-vitro oxidation of ascorbic acid and adrenaline. But, no evidence has been offered by them so far that vitamin P factors act in the same manner in vivo."

Other Theories of How Vitamins P Act

Javillier and Lavollay (14) do mention the possibility of a direct action on capillary walls rather than through an adrenalin mediated medium. Haley, Clark and Geissmann (26) have reported a study to see whether compounds known to have vitamin P activity would show any microscopically visible vasomotor effects on the intact mammalian capillary bed when applied topically in the absence of extrinsic adrenalin (epinephrine). They used the capillary bed preparation of Chambers and Zweifach.

They found rutin, rutin sodium acid succinate, hesperidin, hesperidin sodium acid succinate, methylated hesperidin chalcone, esculin, and adrenochrome inert in the concentrations used. The sodium acid phthalates of rutin and hesperidin were slightly active vaso-constrictors. The catechins, on the other hand including d-catechins, epimerized d-catechins, and especially l-catechin in doses as low as 0.0001 mcg. or less, were highly active vaso-constrictors. But while Parrot et al reported d-epicatechin to be the most active vitamin P they tested, in the tests of Haley et al epimerized d-catechins were no more active vaso-constrictors than pure d-catechin. L-catechin was as active as d-epicatechin. They drew this conclusion:

> "It is possible that some of the biological and chemical effects ascribed to vitamin P can be explained on the basis of a vaso-constrictor effect on the capillaries rather than an indirect effect on these vessels through an epinephrine-sparing action or by a direct action on capillary walls."

If these tests were valid for detection of such activity, they appear to eliminate from such method of action compounds that have been found actually effective in controlling capillary fragility and permeability.

Puig-y-Muset has also (27) reviewed the possible methods of action of vitamin P. He divides his consideration under two heads: Possible chemical or physico-chemical action and enzymatic action.

Chemical and Physico-chemical Action

It is suggested that vitamin P might affect the adrenalin indirectly. One such way would be its part in the metabolism of tyrosine, generally accepted as a precursor of adrenaline.

Parrot and Cotereau (28) showed that certain vitamin P compounds to be not only antioxidant for adrenalin but also for ascorbic acid. Lan and Sealock (29) have reported that ascorbic acid is necessary for the metabolism of tyrosine. They showed that normal guinea pig liver slices show an increased uptake of oxygen when l-tyrosine is present as the substrate, while slices from scorbutic animals are unable to metabolize the amino acid. Also the addition of ascorbic acid in vitro or in vivo restores that ability. By protecting the ascorbic acid from oxidation, vitamins P might function as a sort of vitamin C_2, and thus permit conversion of tyrosine to adrenalin and sympathin. The literature on ascorbic acid as related to adrenalin has been reviewed by Ekman (30).

Muset (31) has also suggested another possible chemical action of vitamins P on the intercellular cement and its pores. He postulates that the intercellular cement substance of the capillary endothelium may be fixed or tanned by vitamins P and thus rendered less fragile or permeable. Catechins are known to precipitate the hide powder of collagen and also to precipitate gelatin. While hesperidin and eriodictyol do not do so they are absorbed by hide powder collagen. This idea of direct action of vitamins P on intercellular cement substance is also suggested by Sokoloff and Redd (25) who state that their microchemical investigations:

> "Suggest the presence of vitamin P factors in the intercellular cement and that in the case of a greatly increased capillary fragility the vitamin P factors can no longer be detected in the intercellular cement pores."

Enzymatic Action

Another possibility canvassed by Muset is based on views that there are intracellular enzymes called cathepsins which are activated by reducing substances, inactivated by mild oxidation, and reactivated by reduction. It is known that in cases of ascorbic acid deficiency there are serious alterations of the cathepsins. In scurvy, the cathepsins are mobilized and may hydrolyze the endothelial cement substance, an action leading to capillary fragility, blood extravasation and hemorrhage.

It is also known that the injection of pepsin and trypsin into animals is often accompanied by hemorrhages simulating scurvy. It has been further demonstrated that when pepsin was injected into guinea pigs which had previously received 30 mcg. of a vitamin P preparation, the animals survived longer than the controls and on autopsy showed less extensive hemorrhages.

According to this viewpoint vitamins P might be considered to control bleeding as an anti-enzyme and the effect might be due to the phenolic component.

Clark, however, comments that studies in his laboratory have failed to confirm this viewpoint. He used ficin from fig sap instead of pepsin because it was more active at neutrality. He does admit that protection against lethal doses of pepsin, trypsin or papain might be obtained by pretreatment with vitamins P if the doses were carefully determined.

Little has actually been done to clarify the enzyme effects of these vitamin P substances. Huszak (32) reported that flavonoid compounds acted as hydrogen transporters in a peroxidase-ascorbic acid oxidase oxidation of ascorbic acid, and that the ortho-diphenolic grouping appeared necessary. The flavanoid compounds were more active than pyrogallol, hydroquinone and pyrocatechol. However this system has not been demonstrated in animals.

Lavollay and Neumann (33) found that the system H_2O_2-peroxidaseflavanoid-adrenalin proceeded at a faster rate than without the flavonoid, but unlike Huszak found the o-dihydroxy group unnecessary. Lavollay and Croix (34) found that epimerized d-catechin inhibited the oxidation of adrenalin by the system cytochrome c-cytochrome oxidase. This reaction appeared similar to the simple iron-catalyzed auto-oxidation of epinephrine which is inhibited by o-dihydroxy phenolic vitamin P compounds.

Summary

It is evident from the preceding discussion that an extensive number of compounds have vitamin P effect on capillary fragility or permeability. Also, to date, no single theory of how they produce their effect has been established. In fact the very idea of a vitamin P has been questioned. It will be recalled that Chambers and Zweifach (18) questioned the validity of assuming a single permeability factor such as a hormone or vitamin and Javillier has also questioned the classification of various fragility and permeability factors under a single vitamin P heading.

In Section III is reviewed the studies of vitamin P substances and human and animal response and the question as to whether they should be regarded as nutrient essential, vitamins, or simply pharmaceuticals.

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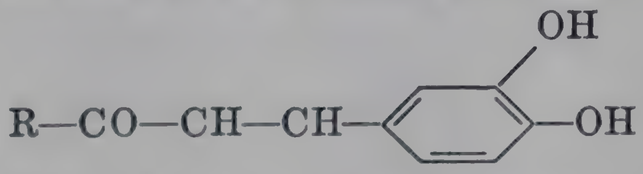
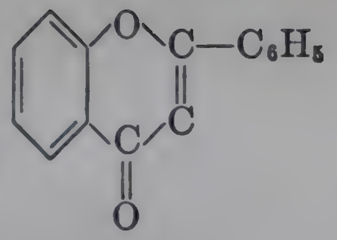
SECTION II. FORMS AND CHEMISTRY OF VITAMINS P

As stated in Section I Szent-Györgyi believed that the activity found in his "vitaprik" and in citrin derived from a flavone compound. Hence, the first compounds to be studied as potential vitamins P were the flavanone glucosides. In these compounds a rhamnose-glucose fraction constitutes the sugar component attached to a flavanoid nucleus. The aglucones of the glucosides studied were hesperitin, quercitin and eriodictin. The structure of these compounds is shown in figs. 36 and 37. Later the French workers added certain catechins to the Vitamin P group, notably the epimer of d-catechin.

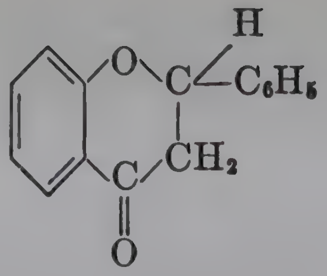
Uncertain still is the structure of the molecule that endows a vitamin P with its capillary fragility and permeability control. Clark and Geissman (1) reported in 1948 that many flavanones, flavanols, flavones, chalcones and other miscellaneous ortho-dihydroxy-phenolic compounds have been examined by them for vitamin P activity and the results indicated to them that high activity depends on the following structure:



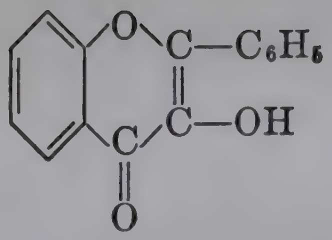
In this grouping 'R' can be among other things, another hydroxy-phenol if the OH groups are placed properly. They also state that activity is probably related to the reducing powers of the compounds, quinone formation and metal complexing or chelating capacity. Clark states that the metal complexing activity is strongly suggested from a molecular viewpoint. This is not a new concept since the flavonols have long been used as dyes and are known to mordant with iron, chromium, aluminum etc.



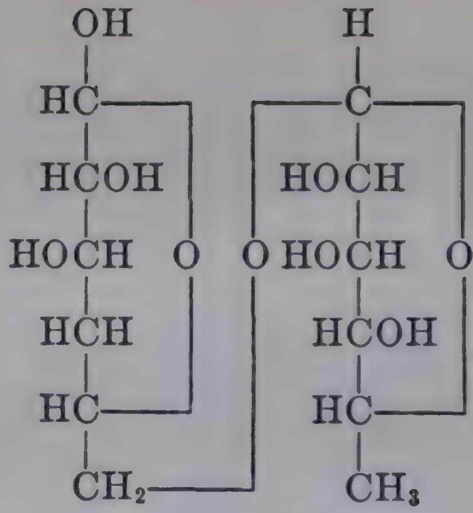
Flavone



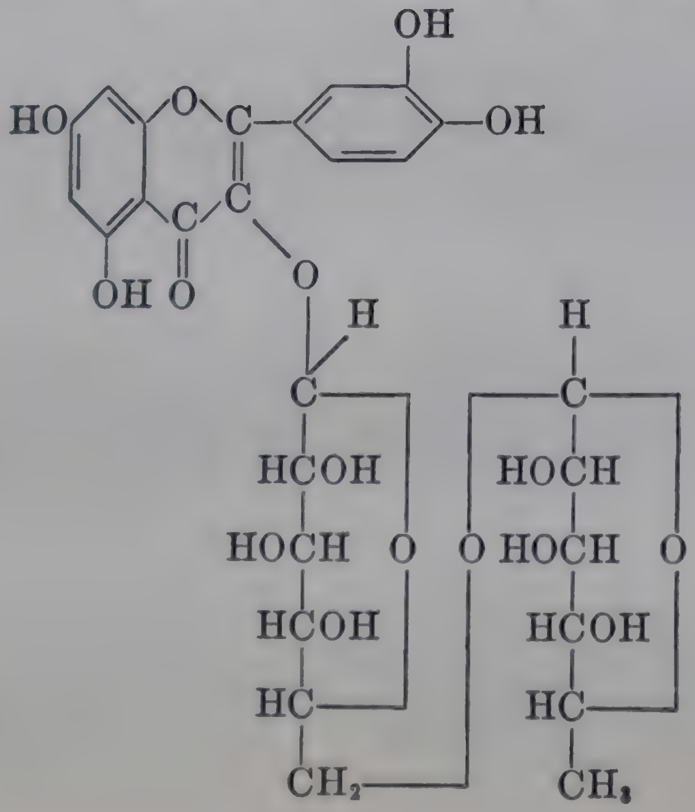
Flavanone



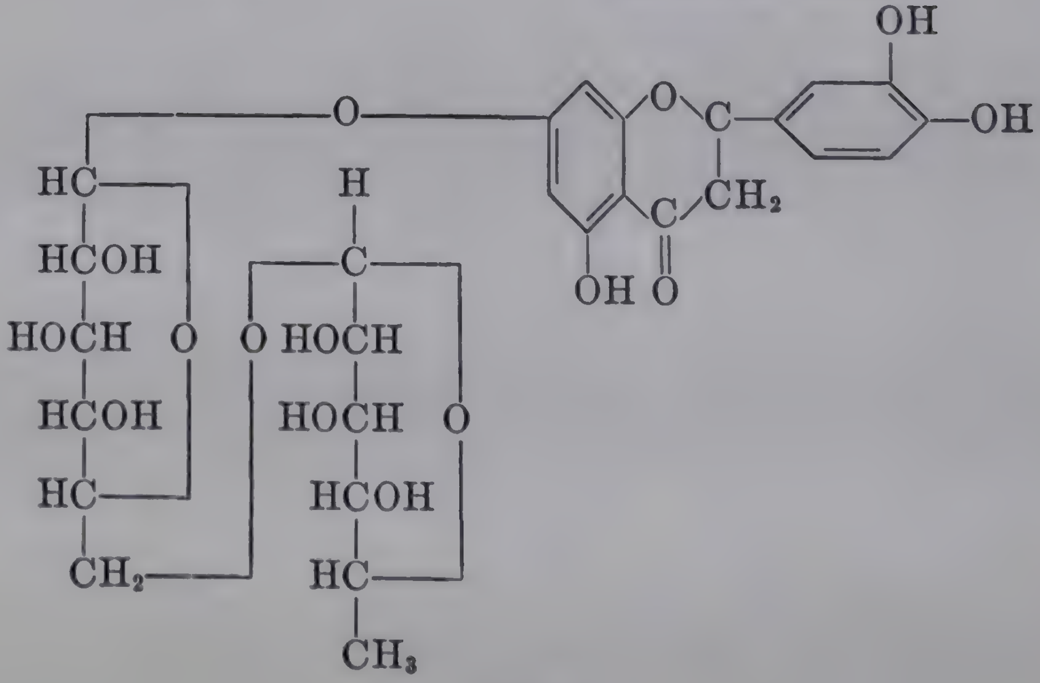
Flavonol



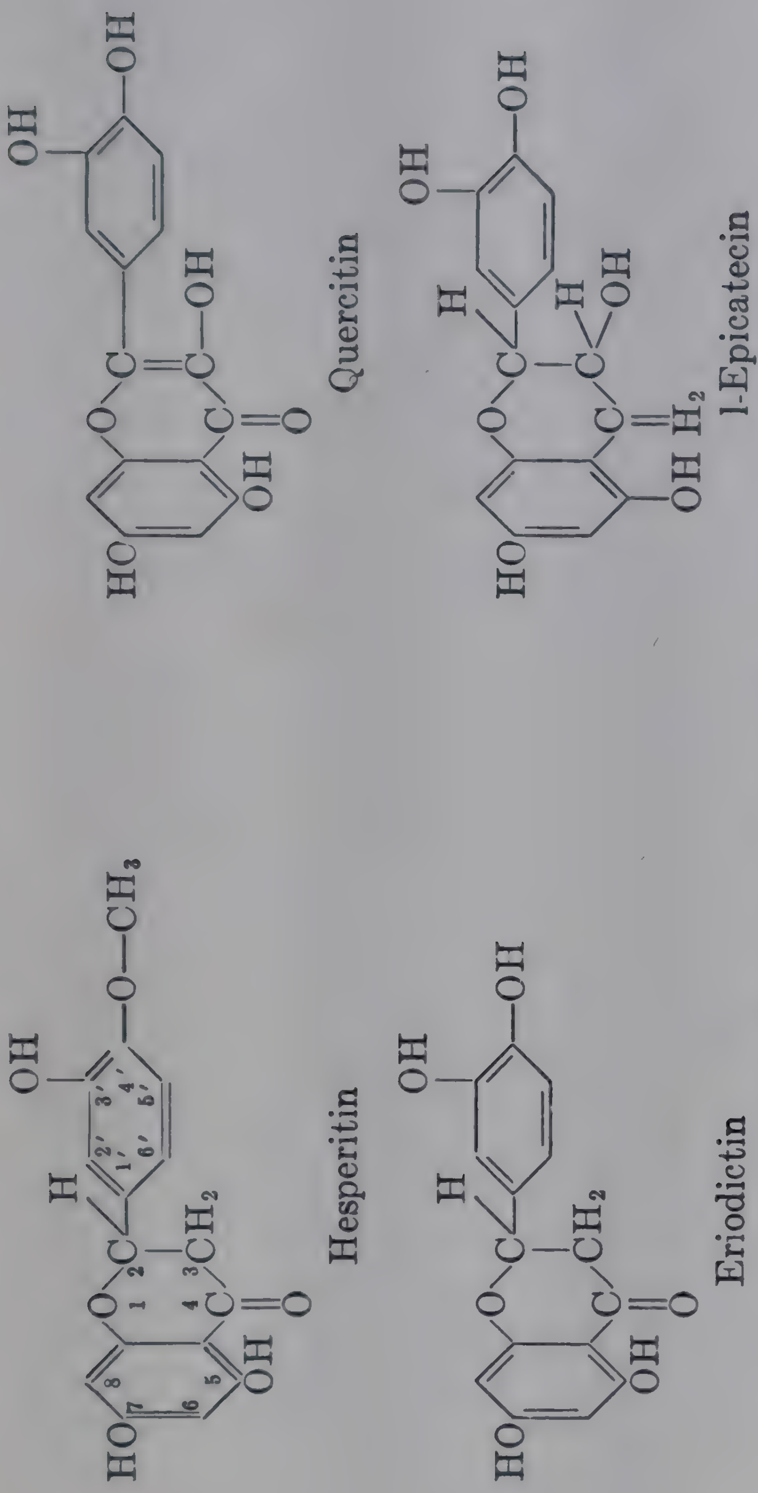
Rhamnose-Glucose



Rutin



Citrin
(Eriodictin-glucoside)



Hesperitin

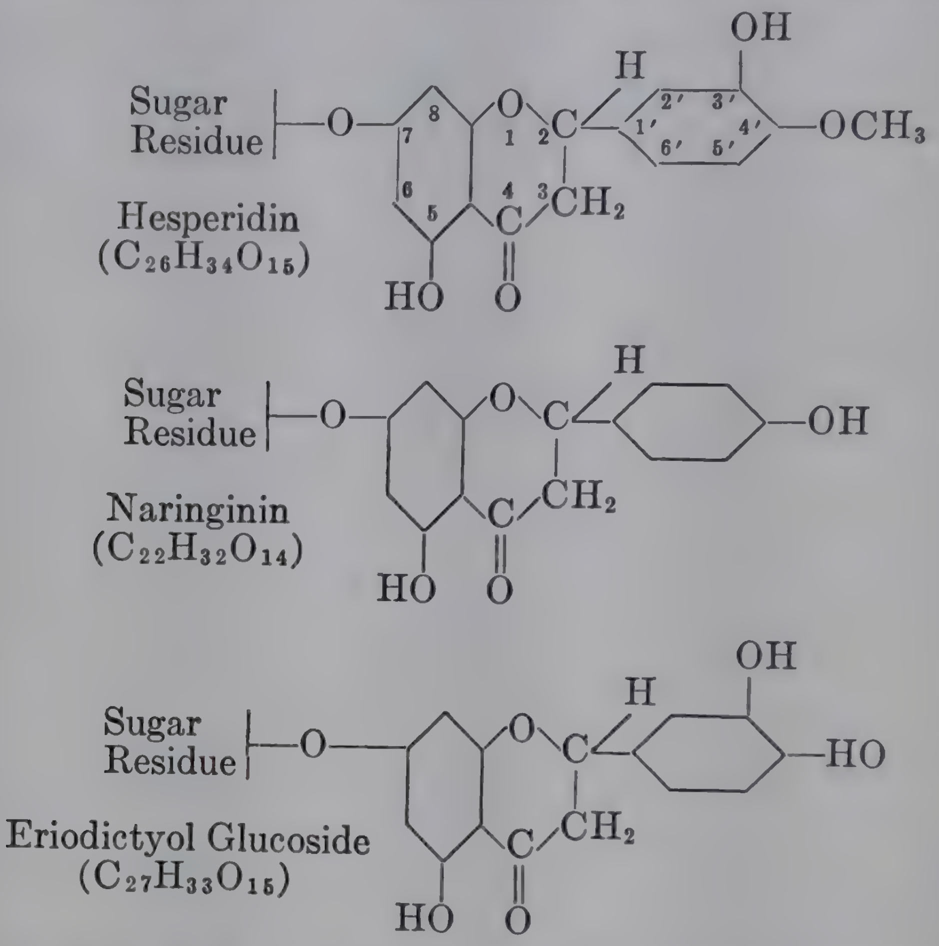
Quercitin

Eriodictin

l-Epicatecin

FIG. 36. STRUCTURES RELATED TO VITAMINS P

Karrer (2) has noted that the colors of yellow flowers, roots and woods are due to various dyes that fall into two groups. One group composes the so-called lipochromes (carotenoids and xanthphylls); the other comprises chiefly different hydroxy flavones and flavonols. But a large number of plants also contain crystalline colorless compounds which are to be regarded as hydrogenated flavonols or hydrogenated anthocyanidins. The catechins are very probably the parent substances of a widely occurring series of amorphous and colloidal red phloriglucinol tannins and tannin reds. All the vitamins P described to date fall within the above groups of plant products.



Hesperidin ($C_{26}H_{34}O_{15}$)

Naringinin ($C_{22}H_{32}O_{14}$)

Eriodictyol Glucoside ($C_{27}H_{33}O_{15}$)

(After Higby R. H., J. of the American Pharmaceutical Association, 30: No 12, p 629 (1941)).

FIG. 37. SOME FLAVANOL GLUCOSIDES

Citrin

The citrin of Szent-Györgyi and the citrus extract used in the early studies of vitamin P action was made from lemons. Lorenz and Arnold (3) have described the following method of preparing a crude vitamin P solution or citrin:

"The whole lemon is sliced in transverse sections approximately one half centimeter thick, covered with water, heated to boiling, and boiled for ten minutes. The solution is then strained and made up to volume. It may be sweetened or otherwise flavored for greater palatability as the taste is quite bitter. An alternative method is extraction of the juice, subsequent grinding of the peel and its extraction as above."

Clark also describes a modification of this preparation which he calls a calcium-flavonate. According to Clark (4) this was:

> "A water-soluble preparation from lemon peel essentially free of water, sugars and hesperidin. The alcohol extract of the fresh peel was precipitated in an alkaline medium with calcium, the precipitate suspended in water and adjusted to an acid pH, reprecipitated by the addition of alcohol, filtered, and the material obtained from the filtrate by evaporation."

Rutin

The quercitin-3-rhamno-glucoside called 'rutin' has been prepared in several ways. Two general types are used: either hot water or alcohol extractions with later crystallization from solution. These methods have been detailed in "The Story of Rutin" (5).

Reactions of Vitamins P

In 1937 St. Huszak (6) utilized the cyanidin reaction of plant pigments as a color test for vitamin P. The cyanidin reaction is based on the phenomenon that pigments of the flavanone type yield with nascent hydrogen a deeply colored cyanidin, the reduction product of the flavanone. St. Huszak also reported that in the presence of eridictyol he got a greenish-brown color with ferric chloride and a dark brownish-black color with ferrous sulfate.

Arcangeli and Trucco (7) have reported using the cyanidin reaction to get a colorimetric determination of vitamin P. Their method, in brief, consists in adding 1 ml. of a solution containing from 0.5–3.0 mg. of vitamin P in MeOH and 0.3 mg. of magnesium. Then cool to 15° C. and add 20% HCl drop by drop until the effervesence ceases (about 30 minutes). Then filter, add HCl to make a volume of 25 ml. and after 30 minutes examine the red color with a Pulfrich photometer, using a standard curve for comparison.

Wilson (8) has described a reaction in an anhydrous system between boric acid and certain flavones and flavanone derivatives which produced a deep yellow color. Working with Szent-Györgyi's original material and Wilson's material from lemon peel, Lorenz and Arnold (3) quantitated the alkali red reaction and suggested a 0.05 N solution of iodine for a standard when a known citrin or vitamin P solution was available.

Occurrence of Vitamins P

Scarborough (9) has reported that P activity is destroyed by boiling a solution in air and that it darkens in the process. Also shaking the solution with animal charcoal decolorizes and destroys potency. In the light, without air contact and at 15–17° C. potency is retained for one or two months. At room temperature in the dark there is a slow loss of potency.

TABLE 59

Distribution of vitamin P in some natural products

(After Scarborough (9) 1945)

Group I. Good sources. Over 100 P.U./100 gms.

		P.U.
Grapes:	black whole fruit	500–1000
	white whole fruit	500–1000
Lemons:	whole fruit	500
	juice	500–750
Oranges:	whole fruit	300–500
	juice	300–600
	peel	200–300
Plum:	blue whole fruit	100–200
Prune:	whole fruit	300–400
Black currant:	puree	200–300

Group II. Moderate sources. About 100 P.U./100 gms.

		P.U.
Grapefruit:	whole fruit	100
Apricot:	dried whole fruit	75–100
Plum:	Victoria whole fruit	50–100
Cherry:	whole fruit	100
Blackberry:	whole fruit	80–100
Bilberry:	whole fruit	100

Group III. Poor sources. Under 100 P.U./100 gms.

	P.U.
Tomato	50
Lettuce and cabbage	under 50
Cauliflower	negligible
Turnip	
Carrot	
Parsnip	
Beetroot	
Potato	

Group IV. Potency of some concentrates

	P.U.
W.S.P.[1] from black currant	300,000/100 gms.
Citrin[2] from whole lemon	65,000/100 gms.
Hipexa[3] from rose hips	20,000–30,000/100 gms.
Hesperidin; recrystallized m.p. 265–266	10,000/100 gms.
Hesperedin; recrystallized m.p. 223–224	2500–5000/100 gms.
Rutin	5000/100 gms.
Rose hip sirup[4]	500/100 gms.

---

[1] Prepared by Dr. A. Pollard of Long Ashton Research Station and for examination by A. L. Bacharach.

[2] Prepared by the method of Szent Gyorgyi (1938) and supplied by Roche Products Ltd.

[3] Prepared and supplied by Dr. F. Wokes, Ovaltine Research Laboratories.

[4] Representation value for commercial preparation bought in the open market.

In 1942 Bacharach and Coates (10) devised a guinea pig test to express potency of vitamin P in what they called provisional units. Using this method Scarborough has (9) presented data on the distribution of vitamin P in certain natural products as shown in Table 59.

Anti-Vitamin P

Parrot and Cotereau (11) have reported the separtion from turnip roots of an anti-vitamin P compound. This compound antagonized the capillary resistance-increasing effect of epimerized d-catechin in guinea pigs. It is apparently polymerized catechin or catechin red.

Summary

Clark states that:

> "There can be no doubt now that there may be a therapeutic effect of these substances, but much careful work must be done to assess the importance of these effects and to elucidate the molecular configurations necessary.

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Section III: Evaluation of Vitamin P in Human and Animal Nutrition

Munro et al (1) have discussed admission of vitamins P as dietary essentials. In their introductory statement they put the situation as follows:

> "From the analysis of the literature it is concluded that diet can sometimes influence capillary strength, as measured by positive and negative pressure tests, but the effect is not consistent. There is very little evidence to associate capillary strength specifically with vitamin C intake, but there is a limited amount of evidence to connect it with vitamin P intake. This raises the question whether or not vitamin P is to be regarded as an essential constituent of the diet. The question can be better answered after consideration of the effects attributed to vitamin P deficiency in man and animals."

And, from their review of such evidence they reached the conclusion that:

> "Claims that lack of vitamin P can lead to a deficiency disease in man are not convincing. It is concluded that there is insufficient evidence to justify the claim that vitamin P is an essential constituent of diet."

Clark (2) comments as follows:

> "In summary, the main nutritional problem remains, to unequivocally establish whether or not a deficiency in these compounds induces any important pathologic, or at least functional lesions such as in the sympathetic nervous system. There can be no doubt now that there may be a therapeutic effect of these substances, but much careful work must be done to assess the importance of these effects and to elucidate the molecular configuration necessary."

In the following pages are reviewed some of the data on vitamin P performance.

Szent-Györgyi's basis for postulating a Vitamin P

As shown in the quotation from Szent-Györgi in Section I (p. 326) he was impressed with the fact that a condition of hemorrhagic diathesis in a friend was corrected by a flavone preparation, and did not respond to ascorbic acid. This suggested that perhaps vitamins P constituted supplementary factors necessary to complete cure of scurvy symptoms. Evidence that ascorbic acid alone does not always eliminate all of the scurvy symptoms and that some factor is present in natural vitamin C sources such as lemon juice was presented by Elmby and Warburg (3) and has been discussed in Chapter XVII (See p. 323.)

Scarborough's Studies

As would be expected, Scarborough's (3) clinical studies of vitamin P were with citrus fruit extracts and on cases of ascorbic acid deficiency. In 1939 he stated that there was no conclusive proof of the existence of vitamin P but, in human subjects suffering from multiple vitamin deficiency, capillary fragility was increased. Orange and lemon juice and certain extracts from them contained a substance, flavanone in character, which increased the resistance of capillary walls to pressure. This substance was not ascorbic acid. Later (4) he reported that, while vitamin P had no effect on subcutaneous hemorrhages, tissue hydration, and other general conditions characteristic of the scorbutic state, it did produce capillary resistance before or after treatment with ascorbic acid and that a deficiency of vitamin P in man might exist even when the ascorbic acid intake was adequate. His studies at least demonstrated that even with adequate intake of ascorbic acid a decrease in capillary resistance could occur and did respond to a type of vitamin P.

We have already cited Bourne's (5) studies to differentiate the capillary resistance effect of vitamin P from that of ascorbic acid. (See p. 328.)

Galmiche's Summary of the Clinical Effects of Vitamins P

French investigators have canvassed the possible relation of vitamin P to various diseases and Galmiche (6) has summarized these studies as shown in Table 60. That record simply shows the cases in which there was positive or negative capillary response to vitamin P therapy. In certain cases there was response, but in general little effect on the malady from which the patient was suffering or any proof that P deficiency caused the malady or that the malady caused the P deficiency effect. In thse studies both citrin and epicatechin were used.

TABLE 60

(After Galmiche (6) 1945)

Diseases treated	Number of cases	By whom	Form of P used	Results
Scurvy, grave though treated with C saturation	1	Donzelot, Bardin, Galmiche, Senecal (1945)	Epicatechin	Cure and recovery
Non-thrombopenic purpura	1	Armentano, Bentsath, Beres, St.Rusznyak, Szent Gyorgyi	Citrin	Capillary resistance raised; no effect on the malady
Non-thrombopenic purpura	1	Jersild (1938)	Citrin	Recovery
Non-thrombopenic purpura	1	Parrot, Lavollay, Sevestre, Galmiche (1944)	Epicatechin	Recovery
Non-thrombopenic purpura	2	Galmiche (1945)	Epicatechin	Recovery
Purpura and edema caused by strain	1	Galmiche (1945)	Epicatechin	Recovery
Hemophilia	1	Galmiche (1945)	Epicatechin	Failure
Cachexia due to starvation	7	Scarborough (1939)	Vitamin P in fruit juices	Improved capillary resistance
Hemorrhagic nephritis	5	Lajos (1937)	Citrin	Recovery
Chronic nephritis	1	Galmiche (1945)	Epicatechin	Resistance improved; no effect on malady
Cardiorenal edema	1	Galmiche and Laederich (1941)	Lemon	Failure
Ascitic cirrhosis	1	Binet and Tanret (1941)	Lemon	Recovery
Ascitic cirrhosis	3	Laederich (1941)	Lemon	Recovery
Ascitic cirrhosis	1	Paris (1942)	Lemon	Recovery
Ascitic cirrhosis	1	Chiray, Lavollay, Maschas (1942)	Flavone extract of orange	Recovery
Ascitic cirrhosis	1	Maschas, Lavollay (1945)	Epicatechin	Recovery
Cryptogenic intestinal hemorrhage	1	Maschas, Lavollay (1945)	Epicatechin	Recovery
Chillblains	1	Galmiche (1945)	Epicatechin	Failure
Eczema	158	Schaber	Citrin	Recovery
Eczema	1	Galmiche (1945)	Epicatechin	Resistance high but unfavorable effect
Arsenical erythrodermia	1	Galmiche (1945)	Epicatechin	Failure
Erythrodermia reticulosis	1	Galmiche (1945)	Epicatechin	Failure
Glaucoma	1	Lavollay, Gallois (1945)	Epicatechin	Recovery
Rheumatic gout	?	Otto (1942)	Citrin	Improvement

Kugelmass' Relation of vitamins P to certain types of Purpura

Kugelmass (7) used citrin tablets prepared after the manner of Lorenz and Arnold in the treatment of four types of purpura (nutritional, allergic, infectious, and mechanical). He claims that benefit ensued in the first three types, but did not show that these purpurae, like scurvy, beri-beri, etc., were the result of a vitamin P deficiency. Increased capillary fragility was an accompaniment of the disease and did respond to citrin therapy.

But, even if these conditions that respond to treatment with vitamins P are not the result of dietary vitamin P deficiency, it is important to know whether vitamins P can be classified as pharmaceuticals useful in treatment and for what diseases. An example of such pharmaceutical value has been cited by Horne and Scarborough (8) in the case of spontaneous hemorrhages that often accompany intolerance to therapy with arsenic and bismuth (anti-syphilitic treatments).

Goldfarb and Psoriasis

Goldfarb (9) tried the effect of citrin on cases of psoriasis. He believed that he found a relationship between psoriasis and injury and between injury and increases in capillary permeability. He used citrin to treat the permeability of psoriasis cases with the following results: of 45 cases treated, 30 improved, 12 showed no change, 3 became worse. The improvement that was noted consisted in the infiltration of the lesions and a lessening of scaling.

Peluse and Gingival Hemorrhage

Peluse (10) has reported a case of a patient with classic telangiectasis distributed about the mouth and face. Gingival hemorrhage first manifested itself at the age of 67. Vitamin K with bile salts and large doses of ascorbic acid failed to control the hemorrhage. Citrin-lemonade controlled the hemorrhage for approximately four weeks, but as soon as the medication was stopped the bleeding recurred. The patient, however, subsequently reported that so long as he continued his citrin intake the bleeding was under control, the gums firm and not sensitive. The citrin appeared to control the bleeding from the telangiectatic area of the gum.

Rutin Therapy

As noted in Section II, rutin is the name of the quercitin-3-rhamnoglucoside. The first report of the clinical use of rutin in this country was by Griffith, Couch and Lindauer (11) in 1944. They tried the effect of rutin on 14 patients with hypertension who also showed increased capillary fragility by the petechial test.

It will be recalled that Robiezniek (12) and Armentano (13) concluded that citrin contained this glucoside, and Armentano attributed to it an effect on blood pressure.

In the clinical tests of Griffith et al, the rutin was given by mouth in capsules starting with 20 mg. twice a day and sometimes increasing to three times a day in a few weeks. So long as the rutin treatment continued it did reduce capillary fragility but did not affect the blood pressure.

In April 1946 Couch and Griffith reported to the American Chemical Society that of 1219 patients studied during the previous 30 months, 21 per cent or 255 patients had capillary fragility. These were treated with 60–120 mg. of rutin per day orally. Follow up was adequate in 173 of these cases and the fragility of 88 per cent of these was returned to normal and remained normal.

They also reported that in 17 out of 20 cases of retinal hemorrhage the capillary fragility became normal, though four suffered further hemorrhage. No toxic effects were noted from the rutin treatment and in these cases the rutin appeared to act as a real cure for the retinal hemorrhage. (See also p. 97.)

Thiocyanate is a recognized drug for the treatment of hypertension. Shanno (14) has reported comparisons of the effect of rutin and thiocyanate. He studied a group of 24 hypertensives. Thirteen of these had increased capillary fragility which improved with rutin. Eleven were treated with thiocyanate and rutin concurrently. Of these seven had normal fragility which was maintained by rutin, two cases on thiocyanate alone developed increased fragility which was made normal by rutin treatment. In one patient with increased fragility, rutin was administered first, followed by thiocyanate after the fragility became normal and was maintained at a normal level by the combined treatment.

There is increasing evidence that, while rutin and other vitamins P are of value in controlling the capillary fragility of hypertensives, they do not correct the hypertension itself. They may be of value in such cases in lessening the danger of hemorrhages.

This viewpoint is supported by Zfass (15). Zfass selected 13 patients who had increased or border-line capillary fragility and 8 of the patients had considerable hypertension. His usual dose of rutin was 20 mg. orally three times a day and this dosage restored the fragility to normal. Also, one of two patients who were treated with thiocyanate developed fragility and rutin restored it to normal.

Zfass comments that the use of thiocyanate in the treatment of hypertensive arteriosclerotic heart disease is dangerous when the patient has increased fragility. The thiocyanate is apparently harmless if the fragility remains normal. Hence, once that fragility is restored to normal by rutin the thiocyante can be used as an adjunct without increasing the fragility. Like the previous investigators he found rutin treatment curative for 3 out of four patients with retinal hemorrhage.

Naghski, Copley and Couch (16) have suggested the possibility of using rutin or similar flavonols to antagonize the hemorrhagic action of dicumaro in vivo. Clark (17), however, found that rutin did not antagonize dicumarolism in rats. He comments that presumably the defect in the vessel walls or their supporting structures which leads to hemorrhage in dicumarolism differs from that leading to the conditions benefited by rutin and other vitamins P. That these facts need not, however, necessarily preclude the simultaneous use of dicumarol and vitamin P for their separate effects was illustrated by the report of McLean and Brabel (18) on the favorable effect of rutin and dicumarol in various retinal vascular disorders.

Vitamin P and Roentgen Ray Treatment

One condition that appears to be definitely responsive to vitamin P therapy is the hemorrhagic syndrome associated with total body roentgen ray irradiation. Griffith et al (19) were the first to report a beneficial effect of rutin following severe X-ray burns of rat extremities. Rekers and Field (20) reported the control of hemorrhagic syndrome and reduction of X-ray mortality with rutin. 50 dogs were exposed to 350 r of total body X-ray irradiation. 25 were given 50 mg. of rutin orally three times daily starting one week prior to the irradiation and continuing through the test period. Of the rutin treated animals only 10 per cent died as compared with 64 per cent of the untreated dogs.

Reviewing these reports, Clark (21) has reported the effect of several flavanoid substances on small animals (rats, mice, guinea pigs) subjected to approximately median lethal doses of total body roentgen irradiation. He used a calcium flavonate prepared from lemon peel. (See p. 339.) Hemorrhagic symptoms of the treated animals were considerably less marked than those of controls. Clark suggests that this finding may give an intact animal assay method for vitamin P substances.

Summary

To date, then, there is no specific syndrome known to be produced in man by dietary vitamin P deficiency, no satisfactory evidence that vitamins P are dietary essentials for either man or animals. There is definite evidence that they may be of value as pharmacologicals in the treatment of capillary fragility whenever it occurs.

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CHAPTER XIX. SOME OTHER POSTULATED VITAMINS

ANIMAL PROTEIN FACTOR[1]

In planning the diets of chicks and for test animals such as the rat increasing evidence appeared that the known vitamins failed to provide all the factors essential to growth. Attempts to raise poultry on all-plant rations, and thus conserve animal protein, indicated that some of the animal proteins contained a water-soluble factor which acted as growth stimulant and was not replaceable by amino acids or known vitamins. Pending identification this factor was tentatively called "animal protein factor".

As early as 1929 Coward et al (1) suggested that yeast was inadequate as a source of water-soluble vitamins and that growth of test animals was improved when their basal diets were supplemented by such materials as fresh milk, wheat embryo, ox muscle, liver, eggs, grass, lettuce or alfalfa meal. Similar conclusions were reported by Guha (2) and in 1932 Mapson (3) demonstrated that certain animal source proteins contained a water-soluble factor essential to the growth and lactation of rats. He called this factor at the time "physin".

Evidence to support this viewpoint came from various investigators (4, 5) and the effect of sources essential to chick growth when on plant protein was carefully studied from 1946 on. Four of these sources were cow manure, the solubles of fish meal, the solubles of distillers grains, and liver extracts.

Hammond (6) in 1942 had reported cow manure to have a beneficial effect on chick growth. Whitson, Titus, Rubin and Bird followed this lead and concentrated on the extraction of the cow manure factor. In 1946 they (7) showed that as the level of soy bean meal in the chick ration was increased from 0 to 40 per cent in increments of 10 per cent, chick growth and also egg hatchability was increased by the addition of cow manure or cow manure extract. Also, the inclusion of 6 per cent dried cow manure, 10 per cent of sardine meal or 10 per cent dry skim milk would correct the detrimental effect of a basal diet containing 35 per cent soy bean meal (8).

Rubin and Bird (9) then proceeded to make concentrates of the manure factor and reported the following experiment: Chicks were placed on a basal diet consisting of 38 per cent yellow corn, 20 per cent barley, 3 per cent alfalfa meal, 35 per cent soy bean meal, 0.6 per cent butyl fermentation residues (containing 0.250 mcg. of B_2 per gram), 1.5 per cent steamed bone meal, 1.0 per cent limestone, 0.7 per cent salt, and 0.2 per cent of an A and D feeding oil (containing 2,000 A and 400 D per gram). To this basal diet they added different supplements which included 5–8 per cent dried cow manure, 5 per cent butyl fermentation residue, 5 per cent yeast, 1 mg. per kilo of body weight of pyracin lactone, pyridoxal HCl or pyridoxamine HCl, 3 per cent Wilson's liver fraction, 1 mg. per kilo of body weight of folic acid, 0.1 per cent choline chloride, and a combination of 0.1 per cent choline chloride and 1 mg. per kilo of body weight of folic acid. Out of all these supplements only the 5–8 per cent cow manure and the Wilson liver fraction produced better growth than the basal ration.

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[1] Funk's streptogenin (J.A.M.A., 132: 304, 1946) may be one of the earliest notations of an animal protein factor.

Between 1946 and 1949 Rubin and Bird and associates (10, 11, 12, 13, 14) succeeded in highly concentrating the cow manure factor and described some of its properties. For example, it was soluble in water, 50 per cent EtOH, 95 per cent alcohol, and insoluble in chloroform and ether; it preciptated from a water extract of dried cow manure at PH3 and it was stable to autoclaving for 2 hours at pH 7, but readily destroyed by autoclaving for one hour in 2N HCl.

They also reported the factor present in hen feces and in hen eggs, more in the yolk than in the white. By use of their concentrate they could counteract the growth-inhibition effect of a 70 per cent soybean meal ration containing no animal protein. This effect was not producible by methionine or any of the known vitamins.

Several laboratories reported that fish meal, dried distillers grain solubles, and crude casein contained the animal protein factor (6, 9, 15, 16, 17, 18, 19, 20). In the case of fish solubles, Robblee et al (16) reported the factor to be soluble in water, 70 per cent EtOh and MeOH, very slightly soluble in 95 per cent EtOH and insoluble in ether and acetone; that it was dialyzable through a cellophane membrane and adsorbed on charcoal, that it was heat stable at 100° C. for 12 hours in a pH range of from 3–9 and not destroyed by enzyme activity.

Novak and Hauge (19) reported the isolation and properties of the factor in dried distillers grains. They separated the factor in non-crystalline form but highly purified state from distillers solubles, rice polish concentrate and liver extract. The product showed maximum absorption at 282 $\mu\mu$ and had definite growth stimulation effect at 2 mcg. per day with maximum effect at 10 mcg. per day.

In the study of the liver source, two lines of research converged. As noted in chapter XVI, while folic acid had hematopoietic effect in cases of pernicious anemia, it did not correct the neurological symptoms while the anti-pernicious anemia factor from liver extract had this ability. Also the liver extract factor did not owe its effect to content of some form of folic acid since assays for folic acid were negative. Hence, the search for identification and isolation of the pure anti-pernicious anemia factor continued, and in 1948 culminated in the isolation of a crystalline product in the Merck laboratories in America (22) and in the Glaxo laboratory in England (23). The ability of this product in very tiny amounts to correct both the hematopoietic and neurological effects in pernicious anemia was soon established (24, 25). This crystalline vitamin has received the name B_{12} and Merck has suggested the name "Cobione" for their commercially distributed product.

From the composition viewpoint special interest attaches to the finding of cobalt as a part of the molecule. According to the Merck chemists, the vitamin appears to be a cobalt coordination complex having six groups about the cobalt atom. Colorimetric estimations showed the presence of 4 per cent cobalt and a probable molecular weight of 1500. Nitrogen and phosphorus are present but no sulfur. The nature of the side groups had not been reported at this writing. The latest data on the composition of vitamin B_{12} at this writing is found in a paper by Brink et al (47). They report on a product obtained from liver and from the culture broth of a grisein-producing strain of Streptomyces griseus. It contained 4.5 per cent of cobalt and its composition is typified by the formula C_{61-64} H_{86-92} $N_{14}O_{13}PCo$ with a minimal molecular weight of 1300. It was optically active; specific rotation 6565 equals −59°. It appeared to be a polyacidic base with an absorption spectrum in aqueous solution characterized by maxima at 2780A (E, 115); 3610A(e, 204); 5500A(E, 63).

Comparison with the folic acid spectrum suggests presence of a pterin-like substance but it contains no amino acids.

English workers (48) have also reported their crystalline B_{12} to have 4 per cent cobalt and a molecular weight of 1600.

Ellis et al (49) also found 4 per cent cobalt in a liver extracted B_{12} with a molecular weight by ebullioscope of 1490 ± 150. They also report that the formulae $C_{62}H_{86-90}$ $N_{14}O_{13}PCo$ and $C_{63}H_{88-92}N_{14}O_{13}PCo$ agree well with the analytical data; that the molecule is not a peptide and that an alkali fusion forms products which react with p-dimethyl-amino benzaldehyde, a characteristic of certain 5 membered nitrogen-containing compounds including pyrroles.

Is Vitamin B_{12} identical with Animal Protein Factor?

There is every indication to date that B_{12} is the animal protein factor. Three separate reports have appeared (26, 27, 28) that show B_{12} to be able to duplicate the effect of animal protein factor on chicks.

Ott, Rickes and Wood (26) compared the effect of B_{12}, liver extract, and fish meal solubles on purified chick rations containing 40–70 per cent soy bean meal as the sole protein source. Quantities of B_{12} as small as 6 mcg. per kilo of diet stimulated chick growth, and the investigators estimated the optimal growth requirement to be less than 30 mcg. of B_{12} per kilo of diet. To achieve the same effect much larger amounts of liver extract and fish solubles were required.

Lillie, Denton and Bird (27) compared the effect of B_{12} with acid precipitate from cow manure and liver extract and also found it to produce the same effects. They state that they estimate their concentrated acid precitate of cow manure to contain the equivalent of 5.8 mcg. of B_{12}.

Nichol, Cravens, Dietrich and Elvehjem (28) have reported a comparison of B_{12} with Lillie's liver extract (reticulogen) and condensed fish solubles. They summarize their results in the statement: "Pure vitamin B_{12} can replace the animal protein factor activity of condensed fish solubles and injectable liver preparations."

Assay Methods for Animal Protein Factor

In the report just cited (28) use was made of the discovery (29) that animal protein factor would counteract the toxic effect of thyroid and iodinated casein on the growth of rats and mice. In their report, Robblee et al (16) had previously employed this fact as a test to measure the effect of animal protein sources. A method of detection of animal protein factor using mice and this thyroid effect is now established (30).

The discovery of Shorb (31), who found that the micro-organism Lactobacillus lactis Dorner (LLD) required small amounts of B_{12}, was used in the isolation of B_{12}.

With these two test methods available it should soon be possible to evaluate sources of the factor, but such assays are still to be reported. They are needed to settle certain controversial findings that have been reported. For example, in some of the earlier studies it was reported that alfalfa leaf meal appeared to contain the factor. Zucker and Zucker (32) have recently raised the question of "Does animal protein factor occur in plants?" and reported that they could not find it in alfalfa. However, they have also invented the term "Zoopherin" which they define as: "The water soluble factor which restores post-lactation growth to normal in the offspring of dams on all-plant diets".

It may prove that, like folic acid (PGA), B_{12} is only one form of compounds that possess this ability to affect growth, egg hatchability and lactation. Until its molecular structure is established that question and its distribution cannot be settled.

LACTATION FACTORS L1 AND L2

The Japanese investigators have reported isolation of lactation factors from liver (L_1) and from Oriental dry yeast (L_2, 32). In 1945 Nakahara et al (33) reported that 5 mg. per day of anthranilic acid duplicated the effect of L_1 and that 5 mg. per day of adenyl thiomethyl pentose (not adenine) or yeast nucleic acid could duplicate the effect of L_2 (34). In a still later paper, Ugami (35) reported that, while L_2 could not be extracted from liver with acidic 60 per cent alcohol (which did extract it from yeast), liver does contain some L_2.

SUMMER BUTTER FACTOR

As stated in the discussion of vitamin A, Dutch workers reported in 1941 (36) that there was substance in the butter made from the milk of cows on summer rations that stimulated growth and was not present in butter from the milk of cows on winter rations.

Similar findings had been reported by Wisconsin workers (37) with the suggestion that the activity was located in certain fatty acid fractions of butter fat (38). These suggestions led Boer et al (39) to test the growth-promoting activity of the only naturally occurring 18 carbon atom, unsaturated fatty acid, viz. vaccenic acid, an 18 carbon acid with a double bond between the 11th and 12th carbons. Boer et al had found that rats fed a ration containing butter fat grew better than those fed rape seed oil. When 0.1 per cent of vaccenic acid was added to the rape seed oil diet the growth effect approached that of rats on butter fat. They therefore postulated vaccenic acid as the butter growth factor (40).

Further investigation has failed to confirm this postulate. Deuel et al (41) could get no enhanced growth effect when vaccenic acid was added to rape seed oil or cotton seed oil diets. The Dutch workers themselves (42) have now reported that when vaccenic acid was made in sufficient quantity and purity by partial hydrogenation of tung oil and china wood oil it had no nutritive effect, and that the growth effect of summer butter could not have been due to its vaccenic acid content.

That vaccenic acid is not the growth factor in butter fat has been further demonstrated when Nath et al (43) got no increase in rat growth on diets of corn oil or olive oil supplemented with vaccenic acid.

The problem of the summer butter factor is still unsolved at this writing but the Wisconsin investigators (43) make the following statement:

> "The fact still remains that there is something present in butter fat at certain seasons of the year which may be either a definite chemical compound or, more likely, a fraction rich in certain favorable fatty acids. This factor or factors concentrated by cold fractionation from acetone has greater stimulating properties than corn oil or the butter fat itself."

VITAMIN B_{14}

1949 has seen report of still another hematopoietic factor. Norris and Majnarich (44) have reported the isolation of what they call vitamin B_{14} from human urine (33 mg. from 100 liters of urine) in the forms of balls of small brown crystals. These crystals showed by analysis 19.6 per cent nitrogen, 4 per cent phosphorus, but no sulfur or cobalt. Its activity was tested on bone marrow culture and found 5,000,000 times as effective as xanthopterin.

Mention has already been made (p. 291) of the xanthopterin studies of these investigators. Their vitamin B_{14} can apparently be formed from xanthopterin by the action of enzymes from several sources. For example Norris and Majnarich have reported that the action of milk xanthine oxidase on xanthopterin and on folic acid produces substances with marked activity in the proliferation of red and nucleated cells in the bone marrow culture technic. This was particularly evident in the case of folic acid which it may be recalled was inactive in the bone marrow test.

They also studied the effect of liver homogenate on folic acid and pterins. Xanthopterin in optimum amount had a high activity before incubation and this activity was only slightly increased by incubation. Folic acid and teropterin in contrast since they were wholly inactive before incubation acquired great activity in the process. Marked increase in activity of xanthopterin and folic acid also occurred by combination with gastric mucosa extract, an activity similar to that of vitamin B_{14}.

These studies along with those of Berk (50) that oral administration of B_{12} to cases of pernicious anemia failed to produce satisfactory response until administered with gastric juice incites interest anew in the intrinsic and extrinsic theories of Castle. Berk interpreted his result to increased absorption of B_{12} by the intrinsic factor in the gastric juice but the idea of direct conversion of dietary factors to hematopoietic substances is possible by intrinsic factor and certainly suggested by Norris and Majnarich's findings. They suggest that extrinsic factor may be one or more substances of the type of xanthopterin folic acid, pteroyl derivatives etc.; that intrinsic factor is in the nature of an enzyme; that B_{14} is an example of the interaction of the two factors.

VITAMIN T

From Austria comes a report of a growth activator and protein assimilator which the investigator (45) calls vitamin T. According to these reports the vitamin was extracted from yeasts and molds and from insects that feed on such microorganisms. Of these concentrates which the author calls "torutilin" from Torulopsis utilis, "temitin" from termites, "penicin" from Penicillium after removal of the antibiotic properties, torutilin proved the most potent, but penicin plus B vitamins matched the torutilin effect. Goetsch states that the factor accelerated the growth of mice, salamanders, frogs, ants, drosophila and molds. In insects it produced abnormally oversize growth. He attributes the effect to stimulation of portein assimilation, increased oxygen consumption and mobilization of reserve substances.

It seems quite different from a factor that Fraenkel et al (46) report to be required by the meal worm (Tenebrio molitor) for growth, and whic they have tentatively labelled B_T. Their factor appears related to th Will's factor and the S factor, suggesting an anti-anemic function. It is no a folic acid or a folic acid conjugate.

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